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MD THESIS

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CELLULAR CERVICAL IMMUNE RESPONSE IN WOMEN WITH HISTORY OF PREVIOUS PRETERM LABOUR

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May 2009

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Thesis submitted in accordance with the requirements of the University of Liverpool

For the degree of Doctor of Medicine

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May 2009

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I declare that this thesis entitled

CELLULAR CERVICAL IMMUNE RESPONSE IN WOMEN WITH HISTORY OF PREVIOUS PRETERM LABOUR

is entirely my own work. It has not been accepted for any degree and has not been submitted for any other degree

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ABSTRACT

Preterm births comprise 6–10% of all births in Western countries. They are responsible for 75–90% of all neonatal deaths not due to congenital anomalies, and for 50% of childhood neurological disabilities.

The exact mechanisms leading to the initiation of preterm labour are not yet known. However, some of the processes involved in preterm labour have been described.

Leukocytes are thought to migrate into the cervix before labour. This cervical leukocyte infiltrate is composed principally of granulocytes and macrophages which are thought to secrete inflammatory cytokines, which in turn initiate labour.

However, previous investigators have studied the cervix after delivery not prior to the onset labour, or found a much greater increase in cervical leukocytes postpartum. Thus, the cervical lymphocyte infiltrate previously described could be an effect rather than a cause of labour. Hence the question remains as to whether cervical leukocytes' infiltration is a causative factor in initiating preterm labour.

In order to test the hypothesis that an increase in cervical leukocyte populations are associated with the onset of recurrent idiopathic preterm labour we adapted a previously described, non-traumatic, method of sampling the epithelium of the cervix at 12-16 weeks gestation and again eight weeks later using a fine cervical cytobrush.

The aim of the present study was to investigate the cervical epithelial leukocyte population in women with a history of spontaneous idiopathic preterm labour. Initially we optimised the timing of sampling then tested the hypothesis that recurrent preterm labour is associated with an excessive cervical epithelial leukocytosis prior to the onset of labour. We also evaluated the cervical leukocyte response to abnormal genital tract pathogens.

One hundred and six women were recruited from an antenatal clinic dedicated to the care of women with a history of pre-term labour at the Liverpool Women's NHS Foundation Trust, Liverpool.
All women had investigations for Bacterial Vaginosis (BV), Gardnerella vaginalis (GV),Trichomonas Vaginalis (TY), Yeasts, Group B Haemolytic Streptococcus (BHEM), Chlamydia, Ureoplasma and Mycoplasma. In addition, serial transvaginal ultrasonography of their cervix, and the mode and gestation at delivery recorded.

Leukocyte sub-populations were examined using immunocytochemistry and the number of leukocytes per total cell count was calculated.

The population of leukocytes compared to epithelium cells, varied significantly between women and the CD16+ granulocyte was the most common sub-population in our study group.

We found that there was no significant difference in leukocyte populations in the cervical mucus of our high-risk pregnant women during the first and second trimester of the pregnancy and cervical leukocytosis was not a prologue to cervical shortening and funnelling.

Our study did find that a specific leukocyte subtype, the macrophage (CD14+), was less common ($p < 0.01$) in the cervical epithelium in the early second trimester of pregnancy among high risk women who subsequently had a recurrent spontaneous preterm labour and delivery compared to those that delivered after 35 weeks of gestation.

We found that there was no significant difference in leukocyte populations between the cervical mucus samples obtained from the women with abnormal genital tact pathogens and in the samples from the women who didn't have any vaginal infection.

The results of this prospective observational study prompt us to speculate that cervical epithelial macrophages may serve to prevent recurrent preterm labour, possibly by preventing ascending infection.

ACKNOWLEDGEMENTS

This thesis is the final result of the collaboration of many people and I would like to state my gratefully acknowledge the contribution of others to this work.

First of all, I would like to thank my supervisor Dr S. Quenby for her great support and guidance throughout the duration of this project. I believe that her support was the most important element of my success.

Also, I would like to thank Dr G. Vince for her help every time it was asked for.

I am also grateful to Paul Nickson and Michelle Bates for technical support with immunocytochemistry and to Lorna Wood for clinical support during the clinic sessions.

Moreover I am grateful to clinical colleagues for referring patients to our clinic and to all the staff members of Liverpool Women's Hospital.

I am also grateful to my parents Athanasios and Stella, for giving me the opportunity of studying at Liverpool University.

Lastly, but in no way the least, I thank Amalia for her help, support and understanding during my studies. Without her it would not have been possible to achieve my goals.

ABBREVIATIONS

APAAP	Alkaline phoshatase anti-alkaline phosphatase
BHEM	Group B haemolytic Streptococcus
BSA	Bovine serum albumin
BMI	Body mass index
BV	Bacterial Vaginosis
CIN	Cervical intra-epithelial neoplasia
CT	Chlamydia trachomatis
g	grams
GBS	Group B streptococcus
GCSF	Granulocyte colony stimulating factor
GM-CSF	Granulocyte macrophage colony-stimulating factor
GV	Gardnerella vaginalis
HAV	Hepatitis
HPV	Human papillomavirus
HSV	Herpes Simplex Virus
IVF	In vitro fertilisation

Ig	immunoglobulin
LLETZ	Large loop excision of the transformation zone
M	Molar
MHC	Major histocompatibility complex
ml	millilitre
mm	millimetre
µl	micro litre
NaCl	Sodium chloride
nm	nano metre
NHS	National Health Service
NICHD	National Institute of Child Health and Human Development
NK cells	Natural killer cells
NOS	Nitrous Oxide Synthase
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PLA_2	Phospholipase A_2
PMNLs	Polymorphic nuclear leucocytes

PPTL	Previous preterm labour
PPROM	Preterm premature rupture of membranes
PG	prostaglandin
PROM	Prelabour rupture of membranes
PTD	Preterm delivery
PTL	Preterm labour
RCOG	Royal College of Obstetricians and Gynaecologists
SCJ	Squamocolumnar junction
T_h cells	T-helper cells
T_c cells	Cytotoxic T cells
T_{reg} cells	Regulatory T cells
TNF	Tumour necrosis factor
TV	Trichomonas vaginalis
Uu	Ureaplasma urealyticum

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CHAPTER 1 INTRODUCTION

Chapter 1

1.1 Background

A preterm delivery, as defined by the World Health Organization, is one that occurs at less than 37 and more than 20 weeks' gestational age. Preterm births comprise 6–10% of all births in Western countries and account for more than two-thirds of all perinatal deaths. The annual number of preterm births worldwide is estimated to be approximately 13 million (Hall et al., 1997). The incidence of spontaneous preterm birth before 37 weeks is approximately 7–11% of pregnancies and before 34 weeks in 3–7% of pregnancies (Honest, et al., 2003). The incidence of preterm birth has not decreased over the years despite major improvements in medical, especially perinatal care facilities, the socio-economic status of the population in developed countries, and extensive medical research. In fact, in most industrialized countries it has increased slightly (Goldenberg 2002).

1.2 Outcome of preterm birth

Prematurity remains a leading cause of neonatal morbidity and mortality in developed countries, accounting for 60–80% of deaths of infants without congenital anomalies. As the risk of neonatal morbidity and mortality near term is low, greater attention is now being focused on early preterm birth (32 weeks' gestation). Although births in this gestational age group represent 1% to 2% of all deliveries, they account for nearly 50% of all long term neurological morbidity and about 60% of perinatal mortality (Goldenberg 2002). Preterm birth is responsible for 75–90% of all neonatal deaths not due to congenital anomalies, and for 50% of childhood neurological disabilities (Iams et al., 2003, Hack et al., 1999). In the neonatal period, preterm infants are 40 times more likely to die than term infants, and they are at increased risk of infant morbidity (Buekens 1994, Chan et al., 2001, Hack et al., 2002). Perinatal morbidity and mortality for infants born without congenital abnormalities is primarily dependent on gestation. Neonatal mortality increases from about 2% for infants born at 32 weeks to more than 90% for those infants born at 23 weeks (To et al., 2004). The EPICure study (Wood, et al., 2000, Costeloe, et al., 2000), investigated the outcome of infants born at less than 25 completed gestational weeks. The percentage of singleton infants who survived and were discharged from the NICU increased from 23% at 23 weeks to 38% at 24 weeks and 54% at 25 weeks. The incidence of disability in infants born at less than 25 completed weeks of gestation was 48%, and 23% of them had severe disability. The highest perinatal mortality occurs at the extremes of prematurity (24–26 weeks); by around 34 weeks, the mortality begins to approximate to that of a term infant. The earlier the PTD, the greater the morbidity related to prematurity, which includes cerebral palsy, neurodevelopmental delay, blindness, deafness and chronic lung disease. Epidemiological studies of preterm labour vary in terms of categorisation but in general terms labour at <24 weeks is considered pre-viable, <28 weeks is extremely preterm, 28–31 weeks very preterm and 32–36 weeks mildly (or moderately) preterm (Moutquin 2003). Sequel of preterm

birth includes short-term morbidities associated with preterm delivery such as: respiratory distress syndrome, intraventricular hemorrhage, periventricular leukomalacia, necrotizing enterocolitis, bronchopulmonary dysplasia, sepsis, and patent ductus arteriosus. Long-term morbidities include cerebral palsy, mental retardation, retinopathy of prematurity, cerebral palsy, cognitive impairment, blindness, deafness, respiratory illness, complications of neonatal intensive care, educational disadvantages, and neurosensory impairments continue into adulthood. There is also a social issue because of its association with learning disability, behavioural deficits, impaired cognitive development, etc. The social implications of this disease are magnified when one considers that its sequel begin at birth and last a whole life-time.

Lowering the incidence of preterm birth and associated neonatal morbidity and mortality is a major goal in obstetrics. In recent decades we have witnessed a significant improvement in neonatal survival and serious morbidity rates in preterm infants. Neonatal mortality rates have declined largely because of improved neonatal intensive care and better access to these services. With appropriate medical care, neonatal survival dramatically improves as gestational age progresses, with over 50% of neonates surviving at 25 weeks' gestation, and over 90% surviving by 28 to 29 weeks' gestation. The gestational age of viability is currently around 24 weeks. In line with this, the limit of aggressive intrapartum management should be 24 weeks (Kilpatrick et al., 1997, Rennie et al., 1996). Viability requires the availability of effective clinical interventions for the immediate and longer term care of these infants (Ahner et al.,2001).

Many studies categorise neonatal events and outcomes by birth weight but clearly this has the added complication of including small for gestational age babies who may not be preterm. In addition the presence within a cohort of small for gestational age babies, some of whom may be growth restricted, may confound associations (Zeitlin et al., 2001). Therefore, studies that are defined by gestational age are preferred when exploring the aetiologies and outcomes of preterm labour.

1.3 Classification of preterm labour

Preterm birth could be divided in three categories: medically indicated preterm birth, PPROM and spontaneous preterm birth (Moutquin 2003). Spontaneous preterm labour accounts for 40–50% of all preterm deliveries, rising up to 70% in the population without any risk factor (Morrison et al., 1990), with the remainder resulting from preterm premature rupture of membranes (PPROM) (25–40%) and obstetrically indicated preterm delivery (20 –25%).

The main cause of PPROM is thought to be an infection although sometimes it is preceded by spontaneous preterm labour (Moutquin 2003).

Medically indicated preterm birth could be due to maternal or fetal complications such as severe maternal hypertension/preeclampsia, abruption placentae, severe intrauterine growth restriction or fetal distress. Spontaneous preterm birth is preceded by spontaneous preterm labour which is very difficult to stop (Morrison et al., 1990, The Canadian Preterm Labour Investigators Group, 1992).

Labour symptoms before term are common and difficult to quantify and may or may not progress to the magnitude at which clinicians will diagnose preterm labour and admit a woman to the hospital.

At least half of the women who are diagnosed with threatened premature labour are delivered at term (Gazmararian et al., 2002, Scott et al., 1997).

1.4 Risk Factors for Preterm Labour

The elucidation of the physiology of parturition has proven to be far more complex than anticipated and it is now apparent that preterm labour is not simply "labour before its time." Preterm parturition is now considered as a syndrome caused by multiple pathologic processes able to activate the common terminal pathway of parturition. Several risk factors for preterm birth have been established:

Race and ethnicity

Black women have a prematurity rate of about 16–18%, compared to 7–9% for white women. The relative contribution of various causes of preterm birth differs by ethnic group. For example, preterm labour more commonly leads to preterm birth in white women, whereas Preterm PROM is more common in black women (Meis et al., 1987). Racial differences in preterm birth are a major focus of analysis, but it is increasingly recognised that there may be differences in maternal body size, customs, behaviours, access to services, age distribution, exposure to racism and discrimination and neighbourhood level factors, which make a major contribution to the preterm birth differences attributed to 'race' or 'ethnicity'(Branum et al., 2002).

Anthropometric factors

Both poor and excessive weight gain are associated with an increase in preterm birth, whereas women with a low body mass index (less than 19.8 kg/m2) are at higher risk of preterm delivery (Wen et al., 1990). Low BMI increased the risk of a high level of neutrophils and high vaginal pH measurement (Hatch et al., 2006).

There has been a growing awareness of the adverse pregnancy consequences of maternal obesity; however, preterm labour may be associated with extremes of maternal weight. Three anthropometric

measurements were evaluated as predictors for spontaneous preterm birth in a systematic review (Dietz et al., 2006).

There was a strong association between very low weight gain and very preterm delivery that was of greatest magnitude among underweight women (adjusted OR 9.8, 95% CI 7.0–13.8). Women with very high weight gain had approximately twice the odds of very preterm delivery, regardless of pre-pregnancy BMI.

Socioeconomic factors

Preterm labour is strongly associated with social disadvantage and recurs in successive pregnancies, increasing the burden of healthcare needs and disability on vulnerable families. It is now strongly associated with lower levels of education, lower family income, and not living with a partner.

The role of social class inequality was examined in a Scottish cohort study between 1980 and 2000 (Fairley et al.,2006). The distribution of social class changed over time with greater inequalities by the end of the 1990s than at the start of the 1980s. Over that time period the relative index of inequality (RII) for preterm birth increased from 1.52 (95% CI 1.44–1.61) to 1.75 (95% CI 1.65–1.86).

Physical violence during pregnancy has been evaluated in a number of studies. Severe physical violence was significantly associated with spontaneous preterm labour in a study of 550 participants in North Carolina, USA (Covington et al., 2001).

There is a significant elevated risk of preterm birth associated with both cohabitation (OR 1.29, 95% CI 1.08–1.55) and single motherhood (OR 1.61, 95% CI 1.26–2.07) for women living in European countries where fewer than 20% of births occur outside marriage (Zeitlin J, Saurel-Cubizolles MJ et al., 2002b). In contrast, there is no excess risk associated with marital status when out-of-marriage births are more common (Zeitlin J, Saurel-Cubizolles MJ et al., 2002b).

Working conditions including long hours (>35 hours/ week), standing >2 hours per day, physical strenuous working conditions (Mamelle et al., 1987),

together with high stress jobs with high demands and low control are all associated with an increased preterm birth rate.

Previous obstetric history

There is an increased tendency for preterm birth to recur in subsequent pregnancies. A history of a preterm delivery is one of the most significant risk factors. The recurrence risk of preterm birth in women with a history of preterm delivery ranges from 17% to 40%, and appears to depend on the number of prior preterm deliveries. Mercer (Mercer et al., 1999) reported that women who had a prior preterm delivery had a 2.5-fold increased risk of spontaneous preterm delivery with their next pregnancy. The earlier the gestational age of the prior preterm delivery, the greater the risk for a subsequent early spontaneous preterm delivery. In a US study of over 150,000 consecutive singleton births, if the first pregnancy resulted in a spontaneous preterm birth, then affected women were more likely to deliver preterm spontaneously in the subsequent pregnancy (adjusted OR 3.6, 95% CI 3.2–4.0) (Anath et al.,2006). This applied even if the first pregnancy was a medically indicated preterm birth (OR 1.6, 95% CI 1.3–2.1). The greatest risk of recurrence tended to occur around the same gestational age as the preterm birth in the first pregnancy. The inter-pregnancy interval appears to contribute to the recurrence of preterm birth. Women with shorter inter-pregnancy intervals are at more risk for preterm birth. Basso et al. (1998) found an inter-pregnancy interval of 8 months a risk factor (OR 2.28; CI 1.49–3.48) for preterm birth. Smith et al. (2003) found an increased risk of preterm delivery, both 24–32 weeks (OR 2.2; CI 1.2–4.1) and 33–36 weeks (OR 1.6; CI 1.2–2.2) when the inter-pregnancy interval was shorter than 6 months, even when the first pregnancy was uncomplicated. Women with inter-pregnancy intervals of <12 months were at increased risk of a preterm birth in the subsequent pregnancy (OR 4.2, 95% CI 3.0–6.0) (Hsieh et al., 2005). The risk decreased as the inter-pregnancy interval increased with a relatively low risk at 18–48 months. Similarly, in a case-control study in Israel an interval of <12 months was associated with an increased risk of preterm labour before 34 weeks (Krymko et al., 2004).

Induced abortion has been associated with very preterm delivery (<33 weeks) in the French regional EPIPAGE study (OR 1.5, 95% CI 1.1–2.0) (Moreau et al., 2005) and this was confirmed by the International EUROPOP study across ten European countries (Ancel et al., 2004).

Medical procedures

Prior history of large loop excision of the transformation zone (LLETZ) is associated with an increased risk of preterm labour and delivery in a subsequent pregnancy. This is not related to an increased incidence of PROM (Fahem et al., 2006).

A systematic review of obstetric outcomes after conservative treatment for intraepithelial or early invasive cervical lesions explored the relationship between LLETZ and the risk of preterm delivery. The risk of preterm delivery was increased (RR 1.70, 95% CI 1.24–2.35) as was the risk of premature rupture of the membranes (RR 2.69, 95% CI 1.62–4.46). Cold-knife conization is a risk factor for preterm birth and preterm premature rupture of the membranes and seems to be a risk factor for cervical tears (Klaritsch et al., 2006). For the index pregnancy, genetic amniocentesis performed in the second trimester is associated with an increased risk of both spontaneous and induced preterm delivery (adjusted OR 1.59, 95% CI 1.31–1.92) (Medda et al., 2003).

Fetal-maternal factors

The existence of a male excess among preterm births may shed some light on the aetiology of preterm labour. A study measuring the association between fetal sex and preterm birth in four original datasets reported more males among preterm and very preterm births in most populations, including in vitro fertilisation (IVF) births (OR 1.09–1.24) (Zeitlin J, Saurel-Cubizolles MJ, et al., 2002a).

Multi-fetal pregnancy is a well known contributor to spontaneous preterm labour and accounts for 10% of all preterm births. About 50% of all twin gestations and almost all higher multiple gestations will deliver before 37 completed weeks. The mean gestational age is shorter for twins (36 weeks), triplets (33 weeks), and quadruplets (31 weeks) than it is for singletons (39 weeks) (Goldenberg et al., 2002). Monochorionic placentation is a significant risk factor for preterm twin birth compared with dichorionic placentation (Penava et al., 2004). The impact of increasing numbers of twins and triplets on rates of preterm births has been investigated in an International study (Blondel et al., 2002). In each country (Canada, England and Wales, France, United States) the increase in preterm delivery among multiple births contributed to the rise or stabilisation of the overall rates of preterm delivery. The increase in multi-fetal pregnancy as a result of sub-fertility treatment has an important role to play as twins resulting from sub-fertility treatment have an increased risk of preterm birth compared with naturally conceived twins, albeit confined to mildly preterm birth (34–36 weeks) (OR 1.6, 95% CI 1.3–1.8) (Verstraelen et al., 2005). Extremes in the volume of amniotic fluid, such as hydramnios or oligohydramnios, have been associated with an increased risk of preterm labour (Cunningham et al., 2001).

Systemic infections, such as bacterial pneumonia, pyelonephritis, and acute appendicitis, often lead to increased uterine activity, potentially leading to premature delivery.

Another potentially important clinical risk factor is the presence of uterine contractions. However, because of the large overlap in contraction frequency between those who delivered at term and those who delivered preterm, monitoring contraction frequency was not found to be useful in defining a population at especially high risk for spontaneous preterm birth. So, there is not a linear correlation between the number of contractions per hour and the incidence of preterm birth because of a low sensitivity and low positive predictive value (Iams et al., 2002).

Paternal factors

Extremes of age have also been reported: teenage pregnancy is associated with increased spontaneous preterm labour, while older gravidas (>35 years old) have increased rates of medically induced preterm births (Berkowitz et al., 1998). In an Italian study of women aged 20–29 years the odds of preterm birth increased with paternal age, with the strongest association for very preterm birth (<32 weeks) (Astolfi et al., 2006). The OR among men aged 45–49 years reached 1.91 (95% CI 1.08–3.38). The relationship between paternal and maternal age differences and adverse perinatal outcomes was investigated in a US study of almost 9 million births (Kinzler et al., 2002).

Stress and psychosocial factors

It has been postulated that maternal stress may modulate the pregnant woman's susceptibility to preterm labour. Biobehavioural model has been proposed where maternal stress may act via a neuro-endocrine pathway that activates the maternal–placental–fetal endocrine systems that promote parturition, and/or via an immune/ inflammatory pathway where maternal stress may increase susceptibility to intrauterine and fetal infectious–inflammatory processes (Wadhwa et al., 2001). Numerous reports have highlighted the role of stressful life events (Copper et al., 1996, Whitehead et al., 2002), anxiety (Dayan et al., 2002), nervousness or depression (Peacock et al. 1995), but also psychic functioning (Mamelle et al., 1998), as significant mediators to the onset of preterm labour.

Alcohol consumption during pregnancy is associated with an increased risk of preterm delivery (Kesmodel et al., 2000, Albertsen et al.,2004).Moderate intake defined as three or more drinks a day increased the risk of preterm birth in an Italian study (OR 2.0, 1.8 and 1.9, respectively, for each trimester

of pregnancy) (Parazzini et al., 2003). There appeared to be a dose–response effect in a large Danish study with the highest risk for very preterm delivery among women consuming seven or more drinks per week (RR 3.26, 95% CI 0.80–13.24) (Albertsen et al.,2004).

The relationship between smoking and adverse pregnancy outcomes, including preterm birth, has been described. Smoking is associated with increased risk of maternal (e.g. PPROM, abruptio placentae) and fetal (e.g. intrauterine growth restriction, stillbirth, low birth weight, preterm birth, and perinatal death) adverse outcome (Cnattingius et al., 2004, Lumley et al., 2004, Salihu et al., 2003). In 2002, smoking was reported by 11.4% of all women giving birth in the USA, a decrease of 38%compared to 1990 when 18.4% reported smoking (Smoking during pregnancy—United States 2004). It is considered that smoking has a more significant role in fetal growth restriction than it in preterm delivery. However, women who smoke still have about a 20–30% increase in preterm birth. Smokers were more likely to give birth to very preterm babies in a French study (adjusted OR 1.7, 95% CI 1.3–2.2) (Burguet et al., 2004, Nabet et al., 2005). The relationship between heavy smoking and very preterm birth was complex with a reduced risk of very preterm birth due to gestational hypertension but an increased risk due to other causes for both low to moderate and heavy smoking. Similarly, in a Swedish study, moderate and heavy smokers were at increased risk of preterm labour from all causes (OR 1.9; 95% CI 1.0–3.6, and OR 2.6, 95% CI 1.1–1.6, respectively) (Kyrklund-Blomberg et al., 2005).

Drug use such as cocaine and marijuana has been associated with an increased risk of preterm birth (Holland et al., 1997, Ogunyemi et al., 2004, Andrews et al., 2000). Prenatal cocaine exposure increased the risk of prematurity (OR 2.24) in a study of women in Kentucky, USA (Bada et al., 2005). Tobacco but not marijuana significantly influenced the outcome with a greater aetiological fraction attributable to tobacco. In another study of psychiatric and substance use disorders, each had an independent association with preterm delivery (OR 2.4, 95% CI 2.3–2.6 for substance use) (Kelly RH, Russo J et al., 2002).

Nutrition

A few studies have explored nutritional factors as potential aetiological factors for preterm labour. A small case-control study of Chinese women reported elevated homocysteine and suboptimal vitamin B-12 and B-6 status in association with preterm birth (<37 weeks) (Ronnenberg et al., 2002). Folate status was not associated with preterm birth. These results would need to be confirmed in larger studies. Women who take pre-conceptional multivitamins appear to have a lower risk of both early (<35 weeks) and late (35–36 weeks) preterm birth (OR 0.59, 95% CI 0.12–2.76, and OR 0.40, 95% CI 0.12–1.40, respectively) (Vahratian et al., 2004).

Pollution

Low level air pollution was evaluated in a cohort of 3988 newborn singletons in the city of Kaunas, Lithuania (Maroziene et al., 2002). The risk of preterm birth increased by 25% (adjusted OR 1.25; 95% CI 1.07–1.46) per 10 mg/m3 increase in nitrogen dioxide concentration but there was no association with formaldehyde exposure.

A number of studies from Taiwan have addressed industrial air pollution from the petrochemical, petroleum, cement and thermal power industries (Yang et al., 2004, Tsai et al., 2003). Each study reported a significant association between air pollution and the risk of preterm delivery with varying odds ratios (1.14–1.30).

Genetic Epidemiology

In a review of 18 studies that examined associations between polymorphisms in the maternal or fetal genome and preterm birth, polymorphisms in tumour necrosis factor alpha (TNF-a), a pro-inflammatory

cytokine, showed the most consistent increase in the risk of preterm birth (Crider et al., 2005). A case-control study from Philadelphia, USA reported an increased risk of spontaneous preterm birth with maternal carriage of the TNF- 2 allele and the association was modified by the presence of bacterial vaginosis (OR 6.1, 95% CI 1.9–21.0) (Macones et al., 2004). In a further study, selected TNF haplotypes were associated with spontaneous preterm birth in both African–American and white subjects (Engel et al., 2005). The impact of genetic polymorphisms with prothrombotic and antithrombotic effects on the occurrence of preterm birth has been investigated in a large cohort of very low birth weight infants and their mothers (Hartel et al., 2005). Factor V Leiden, Factor VII, Factor XIII and the prothrombin G20210A mutation were examined. The maternal carrier status of the Factor VII-121del/ins polymorphism (OR 1.7, 95% CI 1.1–2.5) and the lower frequency of infant's factor XIII-Val34Leu polymorphism (OR 0.5, 95% CI 0.3–0.96) were found to be independently associated with preterm delivery. Dihydrofolate reductase (DHFR) is required to convert the folic acid used in supplements and food fortification to the reduced folate forms used for cell division. The DHFR 19-base pair deletion allele was associated with a greater risk of preterm delivery in a US study of 324 women (adjusted OR 3.0, 95% CI 1.0–8.8). In the presence of low dietary folate, the allele may be a risk factor for preterm birth, reflecting a gene–environment interaction.

1.5 Genital Track Infection and PTL

1.5.1. Introduction

There is a growing body of evidence that infection of the decidua, fetal membranes, and amniotic fluid is associated with preterm delivery. Intrauterine infection, which is often subclinical in nature, is a common and important mechanism of disease in preterm parturition. It has been estimated that a minimum of one of every four preterm births occurs to a mother with intra-amniotic infection (Goldenberg et al., 2000). Various microorganisms usually ascend from the vagina/cervix and penetrate the chorioamnion to produce inflammation (chorioamnonitis), and then invade the amniotic cavity to infect the fetus. Amniotic fluid is generally bacteria-free in women not in labour (Romero et al., 2006), so that bacterial detection is likely to be significant. In women with preterm labour and intact membranes, positive cultures are seen in about 13%. In women with preterm labour and intact membranes who deliver preterm, positive cultures are seen in 22%. In those who have premature membrane rupture, positive cultures are seen in 32% at admission and in as many as 75% at the onset of labour.

Consistently, the lower the gestational age at presentation of preterm labour, the higher the frequency of positive amniotic fluid cultures (Watts et al., 1992). Moreover, the lower the gestational age at presentation, the higher the rate of intra-amniotic infection/inflammation (Yoon et al., 2001, Kim et al., 2001) and the greater the risk of fetal systemic inflammation, which increases the likelihood of short- and long-term handicaps (eg, chronic lung disease and cerebral palsy) (Yoon et al., 2000).

Clinical chorioamnionitis complicates 1–5% of term pregnancies, but nearly 25% of preterm deliveries. In a study by Guzick and Winn (1985), histological chorioamnionitis was more common in preterm deliveries than in term ones (32.8% versus 10%). Watts et al (1992) investigated patients in preterm labour and demonstrated that positive amniotic fluid cultures were present in

19% of women with intact membranes with no clinical evidence of intrauterine infection. In women with spontaneous preterm labour, an inverse relationship exists between colonization of the chorioamnion and amniotic fluid and gestational age at delivery. In one study, chorioamnion colonization was associated with 83% of the very early spontaneous preterm births, but played a much less important role in the initiation of parturition at or near term (Goldenberg et al., 2000). Organisms that have been associated with histological chorioamnionitis include Ureaplasma urealyticum, Mycoplasma hominis, Gardnerella vaginalis, peptostreptococci, and Bacteroides species. Ureaplasma urealyticum is the microorganism most frequently isolated from the amniotic fluid of women with both preterm premature rupture of membranes and preterm labour with intact membranes (Yoon et al., 1998). The recovery of this microorganism from the placenta has been associated with an increased perinatal morbidity and mortality (Kundsin et al., 1984).

1.5.2 Pathophysiology of genital tract infection and PTL

The hypothesis that ascending lower genital tract infection leads to preterm labour has been supported by multiple in vivo and in vitro studies (Keelan et al., 2003, Romero et al., 2002, Goldenberg et al., 2000). Microorganisms may gain access to the amniotic cavity and fetus using any of the following pathways: (1) ascending from the vagina/cervix, (2) haematogenous dissemination through the placenta, (3) retrograde seeding from the peritoneal cavity, and (4) accidental introduction during invasive procedures (Romero et al., 1992). The most common pathway of intrauterine infection is the ascending route (Romero et al., 1992). Evidence in support of this includes: (1) histologic chorioamnionitis is more common and severe at the site of membrane rupture than in other locations, such as the placental chorionic plate or umbilical cord (Romero R, Sirtori M, Oyarzun E et al., 1989); (2) in virtually all cases of congenital pneumonia (stillbirths or neonatal), inflammation of the chorioamniotic membranes is present (Benirschke et al., 1959, Blanc et al., 1964) ; (3) bacteria identified in cases of congenital infections are similar to those found in the lower genital tract (Benirschke et al., 1965); and (4) in twin gestations, histologic chorioamnionitis is more

common in the firstborn and has not been demonstrated only in the second twin. It has been suggested that, in the presence of an ascending bacterial infection, organisms pass between membranes and may later reach the amniotic cavity. It is now accepted that microorganisms and their products may ascend from the lower genital tract into the uterus and elicit an intrauterine inflammatory response, which leads to preterm labour and delivery (Hillier et al., 1993). Ascending intrauterine infection is considered to have four stages. Stage I consists of a change in the vaginal/cervical microbial flora or the presence of pathologic organisms in the cervix. Once microorganisms gain access to the intrauterine cavity, they reside in the decidua. A localized inflammatory reaction leads to deciduitis. Microorganisms may then reside in the chorion and amnion. The infection may invade the fetal vessels (choriovasculitis) or proceed through the amnion (amnionitis) into the amniotic cavity, leading to microbial invasion of the amniotic cavity or an intra-amniotic infection. Rupture of the membranes is not a prerequisite for intraamniotic infection, as microorganisms are capable of crossing intact membranes (Galask et al., 1984). Infection/inflammation is the only cause of preterm labour for which the molecular pathophysiology has been well characterized (Romero et al., 2004, Mitchell et al., 1991). The entry of lower genital tract bacteria into the decidua is associated with the recruitment of leukocytes that is followed by cytokine production (Keelan et al., 2003). Bacterial pathogens may release phospholipases, which initiate the formation of arachidonic acid, and hence the arachidonic acid cascade of prostaglandin production. Alternately, these pathogens can release endotoxins, which act on a number of cells, particularly macrophages, to cause prostaglandin or cytokine release. These pro-inflammatory cytokines, which include IL-1, TNF, and IL-6, in turn act on amnion cells or decidual stoma cells to increase expression of enzymes of the prostaglandin biosynthetic pathway. This, in turn, leads to uterine contractions, cervical dilatation, membrane exposure, and greater entry of microbes into the uterine cavity. Cytokines have also been found to stimulate production of matrix metalloproteinases by the chorion and amnion. Matrix metalloproteinases are implicated in both cervical ripening and degradation of the fetal membranes. Lower genital tract bacteria may also act locally, producing enzymes such as sialidase or mucinase,

which may weaken protective cervical mucus and promote bacterial invasion of the upper genital tract (McGregor et al., 1994).

It is also possible that microorganisms normally present within the endometrial cavity (Romero R, Espinoza J et al., 2004b) and recognized by pattern recognition receptors (Abrahams et al., 2004, Elovitz et al., 2003,Wang et al., 2003) induce a proinflammatory response mediated by cytokines such as interleukin IL-1,(Romero R, Brody DT et al., 1989), tumor necrosis factor TNF-alpha (Romero R, Manogue KR et al., 1989 and Romero R, Mazor M et al., 1989), chemokines, IL-8 (Kelly et al., 1992, Esplin et al., 2005), monocyte chemotatic protein-1 (Esplin et al., 2003), thrombin (Elovitz et al., 2001, Elovitz et al., 2000) and initiate a cascade of events that leads to preterm labour and delivery (Baggia et al., 1996, Romero R, Ceska M et al., 1991).

1.5.3 Inflammation and preterm labour

Accumulating evidence supports the idea that inflammation can be detected in the cervix, myometrium, chorioamniotic membranes and amniotic cavity of women in labour. Spontaneous labour at term is associated with the infiltration of inflammatory cells in these tissues and increased production of pro-inflammatory cytokines (IL-1b, IL-6, TNF-a and IL-8) (Keelan et al., 2003, Romero R, Mazor M et al., 1989) and chemokines (granulocyte colony stimulating factor (GCSF), neutrophil attractant/activating peptide-1/IL-8, etc) (Keelan et al., 2003). Recently, using a genome-wide screen, it has been demonstrated that genes involved in the control of inflammation are upregulated in the chorioamniotic membranes after labour at term, even in the absence of histological chorioamnionitis. Of interest is that an inflammatory response as not observed in the maternal circulation of patients with spontaneous labour at term (Haddad et al., 2004).This suggests that an inflammatory process is localised to the membranes/uterus/cervix. These findings may reflect the fact that spontaneous parturition is associated with an increased pro-inflammatory cytokine and chemokine response (Keelan et al., 2003).

1.5.4 Genital infection in PTL with intact membranes

The most common microbial isolates from the amniotic cavity of women with preterm labour and intact membranes are Ureaplasma urealyticum, Fusobacterium species and Mycoplasma hominis (Romero et al., 1988, Hitti et al., 1997). Other microorganisms that have been found in the amniotic fluid include Streptococcus agalactiae, Peptostreptococcus spp., Fusobacterium spp., Staphylococcus aureus, Gardenerella vaginalis, Streptococcus viridans and Bacterioides spp. Occasionally, Lactobacillus spp., Escherichia coli, Enterococcus faecalis, Neisseria gonorrhea and Peptococcus spp. have been encountered. Haemophilus influenzae, Capnocytophaga spp., Stomatococcus spp. and Clostridium spp. were rarely identified (Alanen et al., 1998, Hitti et al., 1997).

In one study, 51.1% of patients between 14 and 24 weeks of gestation presenting with cervical dilatation of 2 cm or more and intact membranes had a positive amniotic fluid culture for microorganisms (Romero et al., 1992). The outcome of patients with microbial invasion was uniformly poor as they developed subsequent complications (rupture of membranes, clinical chorioamnionitis or pregnancy loss). Therefore, infection is frequently associated with an acute cervical incompetence (Mays et al., 2000). Whether intra-amniotic infection is the cause or consequence of cervical dilatation has not been determined. It is possible that clinically silent cervical dilatation with protrusion of the membranes into the vagina leads to a secondary intrauterine infection.

Bacterial Vaginosis

Normal vaginal flora is dominated by lactobacilli, which, by producing lactic acid, keep the pH of the vagina below 4.5, which discourages the growth of other organisms. Under normal conditions of low pH, lactobacilli are able to produce hydrogen peroxide (H_2O_2) which is toxic to bacteria. In normal flora there may be an anaerobe to aerobe ratio of 2:1 or 5:1. Bacterial

vaginosis (BV) is an imbalance of vaginal flora caused by a reduction of the normal lactobacillary bacteria, and a heavy overgrowth of mixed anaerobic flora including Gardnerella vaginalis, Mycoplasma hominis and Mobiluncus species. In bacterial vaginosis, in association with a reduction in the quality and/or quantity of lactobacilli there is a 1,000- fold increase in the growth of other organisms. The anaerobe to aerobe ratio is between 100:1 and 1,000:1. Under circumstances of increased alkalinity, such as bleeding in pregnancy, sexual intercourse or vaginal douching, or under circumstances where antibiotics are used or where there is a change in endocrine status, lactobacilli at high pH are less efficient at producing H_2O_2 which permits the overgrowth of other organisms.In terms of pregnancy, BV has been associated with a 40% increased risk of preterm labour and it occurs in approximately 16% of the untreated female population (Hillier et al., 1995). Fifty percent of women with BV are asymptomatic, but if symptomatic, women may have a grey vaginal discharge with a characteristic 'fishy' odour. It is not associated with vaginal mucosal inflammation and rarely causes a vulval itch. Importantly, the earlier in gestation that BV is detected, the greater is the risk of an adverse outcome (Hillier et al., 1988 and McDonald et al., 1991). Although the association between bacterial vaginosis (BV) and preterm birth has been confirmed in two independent meta-analyses (Pararas et al., 2006 and Galask et al., 1984), the role of BV itself in the pathogenesis of preterm labour and preterm delivery remains unexplained. Two hypotheses may explain the mechanism by which BV plays a role in the onset of PD: first, BV organisms may ascend into the uterus; second and more likely, BV is a marker of intrauterine colonisation by similar organisms (Goldenberg et al., 2000). Organisms associated with bacterial vaginosis, such as Mobiluncus species and anaerobes are able to produce the keto acid, succinate, which is responsible for blunting the chemotactic response and reducing the killing ability of polymorphic nuclear leukocytes (PMNLs). This results in a vicious circle of increasing numbers of organisms without an inflammatory response. Thus, there is a large concentration of potentially pathogenic organisms with no obvious cellular host response. More recently, it has been hypothesized that the risk of prematurity may not depend on the type of vaginal flora alone, but also on the type of immune response that is mounted to control an

infectious process. A good overview of this hypothesis can be found in an article by Romero et al.(2004). This hypothesis also includes evidence that the differences in the types of immune responses may have a genetic explanation. The results of this meta-analysis confirm that BV is significantly associated with adverse pregnancy outcome and that the risk of preterm delivery <37 weeks' gestation, as calculated in the main analysis, is more than doubled in asymptomatic women with BV. There was no significant association between BV and preterm delivery <34 or <32 weeks' gestation. However, only a few studies tested these outcomes.

Hepatitis A (HAV) and Herpes Simplex Virus (HSV) infection and PTL

HAV infection during second and third trimester of pregnancy is associated with a high rate of gestational complications and preterm labour (Elinav et al., 2006).

The infants of women with asymptomatic HSV shedding at the onset of labour who recently acquired genital HSV infections were more likely to be premature than infants of mothers with asymptomatic shedding due to reactivation infections. Among these women with recently acquired first-episode infections, prematurity and LBW were most marked among women with primary genital HSV (Brown Z et al., 1996).

Group B streptococcus (GBS) infection and PTL

It was previously thought that vaginal colonization by Group B streptococcus (GBS) during pregnancy increases the risk of PTL, PPROM and perinatal transmission of the organism (McDonald et al., 1989). Recently new data come with controversial results about the effect of the GBS infection on the outcome of the pregnancy. Kubota found that vaginal colonization with GBS was not associated with preterm labour or ROM in an obstetric population with a low incidence of bacterial vaginosis, despite the presence of lactobacilli-reduced flora in GBS-positive women (Kubota et al., 1998).

Daskalakis et al examined 1,197 pregnant women, to evaluate the relationship between bacterial vaginosis (BV) and group B streptococcal (GBS) colonization in the 2nd trimester of pregnancy and preterm delivery. They found that although BV is a risk factor for preterm delivery, GBS colonization in the 2nd trimester of pregnancy has an inverse correlation with preterm delivery (Daskalakis et al., 2006).

Ureaplasma urealyticum (Uu) and PTL

Ureaplasma urealyticum (Uu) is commonly isolated from various sites during pregnancy, including the cervix, amniotic fluid, decidua, chorioamnion and placenta.

Patients with preterm premature rupture of membranes and microbial invasion of the amniotic cavity with Uu are associated with a robust host inflammatory response in the fetal, amniotic, and maternal compartments (Romero R, Yoon BH et al., 1993a). It has been detected in the amniotic fluid of approximately 6%of patients with preterm labour and is associated with a higher risk of preterm delivery and adverse neonatal outcomes in this setting (Yoon et al., 1998, Yoon et al., 2003). McDonald et al. (1997) reported a colonization rate with Uu of 35% during the midtrimester of pregnancy, and found that colonized women had a risk of preterm delivery almost twice as high and for preterm PROM more than 3-fold than that of women with negative cultures. However, no such association was shown by Carey et al.(1991).Although vaginal colonization with Ureaplasma does not appear to cause preterm labour, this organism may become pathogenic when it gains entry to the upper genital tract.

Chlamydia trachomatis and PTL

Chlamydia trachomatis, an obligate intracellular organism, is found in endocervical cells in infected women. The relationship between infection with Chlamydia trachomatis and prematurity has been examined in cohort as well as case control studies (Gravett et al., 1986, Martin et al., 1982, Andrews WW et al., 2000, Brocklehurst et al., Cochrane Database Syst Rev 2000). The

results have been conflicting. C trachomatis has rarely been detected in the amniotic fluid or membranes of patients with preterm labour or preterm premature rupture of membranes (PPROM), although standard culture media are insufficient to detect this organism. Even when appropriate techniques are used, it has been detected in only 7% of patients with PPROM, and it has never been reported to be present in the fluid of patients with preterm labour and intact membranes. It does not appear to be a common pathogen in clinical chorioamnionitis (Vile et al., 1997, Thomas et al., 1990). The NICHD Preterm Prediction Study found that C. trachomatis infection at 24 weeks, but not 28 weeks, was associated with an increased risk of preterm birth (OR 2.3, 95% CI 1.01–5.03) (Andrews WW et al., 2000). This increased risk of preterm birth remained after adjusting for prior preterm birth. However, a paper by the same group has recently cast doubt on the role of CT in the pathophysiology of preterm delivery. In a further study they showed that amongst women with either bacterial vaginosis or Trichomonas vaginalis, the presence of C. trachomatis was not associated with an increased risk of preterm birth (Andrews et al., 2000). It seems that women with a recent infection, particularly those able to mount a serologic immune response, are more likely to be at risk.

Trichomonas vaginalis and Gonorrhea infection and PTL

The detection of Trichomonas vaginalis was associated with an increased risk of preterm delivery (OR 1.3, 95% CI 1.1–1.4) (Cotch et al., 1997).

Gonorrhea may have a variety of presentations in pregnancy, ranging from asymptomatic infection to symptomatic infection of the cervix, urethra, or rectum. Gonococcal infections may be localized or disseminated in pregnancy. A localized infection may cause an increased risk of preterm labor and preterm rupture of membranes, as well as an increased risk of intrapartum, postpartum and neonatal infection (Donders et al., 1993).

1.5.5 Screening in women who have had previous preterm labour.

There is some evidence that if women who have had a previous preterm birth are screened for abnormal microbial colonisation of the genital tract and treated with antibiotics, some preterm births can be prevented. Morales and colleagues (1994) did a small randomised controlled trial of oral metronidazole in 80 women who had a past history of preterm birth and who had bacterial vaginosis at between 13 and 20 weeks of gestation. Metronidazole reduced the admission rate from 78% to 27%, the preterm birth rate from 39% to 18%, and the rate of PPROM from 33% to 5%. Hauth and colleagues (1995) did a much larger study, which involved 616 women who had previously had preterm birth or who were of low bodyweight and had bacterial vaginosis at 22 weeks' gestation. The women were randomly assigned to either ampicillin plus erythromycin, or placebo. The antibiotics reduced the preterm birth rate from 49% in the placebo group to 31% in the antibiotic group. Further evidence comes from McDonald and colleagues (1997), who did a post-hoc analysis of 424 women who had had a previous preterm birth and who were taking part in a randomised trial of metronidazole in a group of 879 women with a heavy growth of Gardnerella vaginalis. They reported a reduction of the preterm birth rate in this subgroup of women. The summation of the evidence from these trials does suggest that a large trial of screening for abnormal microbial colonisation of the genital tract during the prenatal period in women who have had previous preterm birth and subsequent treatment with antibiotics is needed.

1.5.6 Treatment of genital tract infection

The organisms or infections that have been associated with preterm birth make up the list of candidate infections for treatment antenatally. These include U urealyticum, group B streptococci, N gonorrhoeae, C trachomatis, T vaginalis, bacteriuria, and BV.

Controversy remains as to whether screening and treating all pregnant women for asymptomatic BV and TV will prevent PTD. The largest randomized clinical trial in women with asymptomatic bacterial vaginosis was recently reported by the Network of Maternal–Fetal Medicine Units. Patients with a positive Gram stain and a high pH of vaginal fluid were randomly allocated to metronidazole or placebo. Although treatment with metronidazole reduced the rate of bacterial vaginosis, there was no effect on the occurrence of preterm delivery or other adverse perinatal outcomes (Carey et al., 2000). In a further randomised, double-blind, placebo-controlled tricentre study, 404 women at their first antenatal clinic visit between 13 and 20 weeks gestation, who were diagnosed as having bacterial vaginosis on Gram stain of vaginal secretions, were given a 3-day course of 2% clindamycin vaginal cream or placebo (Lamont et al., 2003). The study showed a 2.2-fold reduction in the incidence of preterm birth and a 3.4-fold reduction in the incidence of preterm birth when treatment was initiated before 16 weeks gestation. Hauth et al. (1995) reported that treatment of women with bacterial vaginosis (and a history of a previous preterm birth) with metronidazole and erythromycin reduces the rate of preterm birth.

Hauth et al .(2001) reported that mid-trimester treatment with metronidazole and azithromycin of women with a history of a previous preterm birth, bacterial vaginosis and a positive fetal fibronectin in cervical/vaginal fluid did not reduce the rate of preterm delivery in patients less than 35 and 32 weeks of gestation. However, two randomized clinical trials and one control trial demonstrated that screening for and treating BV lead to approximately a 50% reduction in preterm birth among women who are at risk for preterm birth

(Morales et al., 1994, Hauth et al., 1995, McGregor et al., 1995). No studies have evaluated the combination of both intravaginal and systemic antibiotics to treat BV in the prevention of PTD.

There are reports of successful pregnancies after antibiotic treatment of women who were colonized by ureaplasmas and who had previously had frequent abortions (Stray-Pedersen et al., 1978, Kundsin et al., 1970). However, to postulate on the basis of this that ureaplasmal infection is a cause of reproductive failure seems inadvisable, as specimens were not examined for other bacteria and the treatment trials were largely uncontrolled or the numbers of patients studied were few.

It appears that M. genitalium, unlike M. hominis, behaves independently of BV and has no part in its development (Keane et al., 2000). In three studies (Lu GC et al., 2001, Labbe AC et al., 2002, Oakeshott et al., 2004), M. genitalium was considered to be an unlikely risk factor in pregnancy outcome, while in a fourth it was reported to be a significant independent risk factor for spontaneous preterm delivery. Clearly, this is an area ripe for further research.

The Vaginal Infections and Prematurity (VIP) study failed to demonstrate clearly that screening and treatment programs for C trachomatis reduce preterm birth. However, this study was limited by a high rate of clearance of Chlamydia in the placebo group, and it appeared that treatment could be beneficial in subgroups of patients with lower rates of spontaneous clearance (Martin et al., 1997).

A large randomized clinical trial in which women with trichomoniasis detected by culture of vaginal fluid were randomly allocated to metronidazole (two 2-grams doses) or placebo concluded that treatment of asymptomatic women did not prevent the rate of preterm delivery (Klebanoff et al., 2001).

In a study by Eschenbach et al. (1991) it was reported that antibiotic treatment (erythromycin) administered at 30 weeks of gestation to women with positive

cervical cultures with Ureoplasma did not result in a reduction in the rates of preterm PROM or PTL.

Routine screening and antenatal treatment of women with GBS does not reduce the risk of PTD. Nonetheless, screening in high-risk groups is recommended in the UK, and universally in the third trimester in the USA and Canada, to initiate prophylactic antibiotic treatment for carriers of GBS in labour. This intervention has been shown to reduce the incidence of GBS-related neonatal morbidity and mortality (Rajesh et al., 2004).

Antibiotic treatment in patients with premature labour

Among patients in preterm labour with intact membranes, several studies have reported the effect of adjunctive use of antibiotics to prolong pregnancy. The largest trial reported today is the ORACLE II. 6295 women in spontaneous preterm labour with intact membranes and without evidence of clinical infection were randomly allocated to receive erythromycin, co-amoxiclav (250 mg amoxicillin and 125 mg clavulanic acid), both, or placebo. The trial demonstrated no improvement either in delaying delivery for 48 hours or in a composite outcome that included neonatal death, chronic lung disease, or cerebral abnormality. One reason that may explain the absence of an effect by antibiotics is that too few cases of threatened preterm delivery are caused by infections in the population studied, which were selected not according to infectious markers but by cervical observations. Antibiotic administration was associated with a significant reduction in the rate of maternal infection (Kenyon et al., 2001b). In a recent meta-analysis from the Cochrane Library, 11 trials that included 7428 women were assessed. The overall use of antibiotics did not decrease preterm birth, delivery within 48 hours, nor perinatal mortality rates. The relative risk for neonatal death in the antibiotic group was 1.52, with the lower bound of the 95% CI at 0.99. Maternal uterine infection was reduced significantly by the use of antibiotics, but this benefit was not seen as a justification for widespread antibiotic use in preterm labour (King et al., 2003). Because not all antibiotics are likely to have the same effect, this analysis also looked at the subgroups of trials. This

analysis concluded that the routine administration of antibiotics to women with preterm labour and intact membranes should not be recommended, because there was no clear improvement in neonatal outcomes and potentially a trend towards increased neonatal deaths (King et al., 2003). Antibiotic use for prevention of perinatal group B streptococcal infection, however, is recommended (Centers for Disease Control Prevention, 2002).

Antibiotic administration in PPROM

Several large randomized trials have investigated this area. In the Maternal-Fetal-Medicine Network study, ampicillin plus erythromycin for 7 days was compared with placebo in patients with PROM from 24 to 32 weeks of gestation (Mercer et al., 1997). Patients who received antibiotics were more likely to remain undelivered when assessed at 2, 7, 14, and 21 days. This prolongation of pregnancy was also accompanied by significant reduction in the composite outcome (defined as neonatal death, respiratory distress syndrome, grade 3 or 4 intraventricular hemorrhage, grade 2 or 3 necrotizing enterocolitis, or neonatal sepsis). Individual components of the composite outcome (such as respiratory distress syndrome and necrotizing enterocolitis) were also reduced significantly. An even larger trial (ORACLE I) demonstrated smaller, but significant benefits in oral antibiotic therapy for 10 days with either erythromycin or amoxicillin clavulanic acid or both, compared with placebo. Perhaps it may be that the smaller effect in the antibiotic groups is attributed to the use of oral antibiotics only or to the inclusion of patients up to 37 weeks of gestation (who are less likely to have infection as the cause of preterm birth) (Kenyon et al., 2001a). The ORACLE Children Study II was designed to investigate whether or not peripartum antibiotics improve health and disability for children at 7 years of age. This study assessed long-term outcomes for 4473 children age 7 years, born in the United Kingdom to 4221 women enrolled in the original ORACLE II trial. Compared to mothers who had received no erythromycin, more children born to mothers who had received any erythromycin had any functional impairment. In contrast, there was no difference in the proportion of children whose mothers had received co-amoxiclavand those whose mothers had not received co-amoxiclav. More

children whose mothers had received any erythromycin presented with bowel disorders. A higher proportion of children whose mothers had received either antibiotic developed cerebral palsy compared to those born to mothers who had not received these antibiotics. The investigators conclude that these findings support the viewpoint that antibiotics are not advisable in SPL without clinical signs of infection (Kenyon et al., 2009).

In 2003, the Cochrane Library updated its meta-analysis of antibiotics for PPROM. Here, 13 trials including 6000 patients were included with the two above trials dominating the analysis. Antibiotics in PPROM had consistent benefits (in contrast to their use in preterm labour with intact membranes). In this analysis, women who were exposed to antibiotics were less likely to deliver within 48 hours, deliver within 7 days, and develop chorioamnionitis, neonatal infection, and neonatal sepsis. Based upon the results of the meta-analysis, the administration of antibiotics to patients with preterm PROM appears justified.

1.6 Risk Factors for Recurrent Preterm Birth

Women who have experienced either PTD, delivery of a low-birth weight infant, second-trimester pregnancy loss or first-trimester pregnancy termination are at increased risk of subsequent PTD. De Haas et al. (1991), in a case-control study, compared 140 women with a preterm delivery and 280 women with a term delivery. Risk factors for preterm delivery were: a history of preterm delivery, smoking during pregnancy, and a history of abortion. Kristensen et al. (1995) found that a previous spontaneous preterm delivery gives a fivefold risk increase in preterm birth in the next pregnancy compared with women with first term delivery. Adams et al. (2000) found in a population-based cohort among almost 123,000 white and 56,000 black women that there is a high association between a preterm delivery in the first pregnancy and recurrence in the next one. For both black and white women, the rate of preterm delivery in the second pregnancy increased as the length of the first pregnancy decreased.

Furthermore, the gestational age of the prior PTD often correlates with the gestation of the subsequent PTD. Cnattingius et al. (1999) found in a cohort study a similar association, that the risk of a very preterm delivery in successive pregnancies is increased primarily among women with a previous very preterm delivery. When the first pregnancy was before 32 weeks' gestation, the risk in the second pregnancy to deliver before 32 weeks was tripled (OR 3.8; CI 2.5–5.4), and when the first pregnancy was between 32 and 37 weeks, the risk of delivery between 32 and 37 weeks was twelve-fold (OR 12.8; 12.0–13.5).

The recurrence risk increases with the number of prior PTDs. Thus, a history of one, two and three prior consecutive spontaneous PTDs is associated with recurrence risks in the next pregnancy of 16–19%, 32–41% and 67%, respectively. Carr-Hill (1985) found the risk of a preterm delivery in the second pregnancy more than tripled in the second pregnancy (from 4.7 to 15.4%),

and more than six times higher with two previous preterm births (from 4.7 to 32%).

Krymko et al. (2004) found that a short interval between pregnancies especially up to 12 months after a preterm birth was associated with fivefold increase in recurrence of preterm birth before 34 weeks. Basso et al. (1998) found similar results with an inter-pregnancy interval after preterm birth of less than 8 months. Mercer et al. (1999) found that a prior spontaneous preterm delivery was associated with an increased risk (21 vs. 8.7%) of a subsequent preterm delivery (relative risk 2.5; 1.9–3.2).

Importantly, women who originally delivered preterm because of twins are not at increased risk of recurrence in a subsequent singleton pregnancy. This may be explained as factors such as uterine overdistension and polhydramnios occurring in twins are unlikely to recur in subsequent singleton pregnancies.

1.7 Histology of the cervix

The cervix is a firm cylindrical structure, around 2 cm long and 1–2 cm wide, and forms the distal component of the uterus. The cervix is actually the lower, narrow portion of the uterus, connected to the uterine body by the uterine isthmus. Its name is derived from the Latin word for "neck." It is cylindrical or conical in shape. Its upper limit is considered to be the internal os, which is an anatomically and histologically defined junction of the more muscular uterine fundus and the denser, more fibrous cervical stroma. The external os is the lower opening of the cervix into the vagina. The passageway between the external os and the endometrial cavity is referred to as the endocervical canal. Its upper limit is the internal os (Droegemuller W et al., in Comprehensive Gynecology 1987).

The stroma of the cervix accounts for most of its mass and shape. The main formed element of the cervical stroma is extracellular connective tissue matrix. The extracellular matrix is made up of type I (66 percent) and type II (33 percent) collagen with a small amount of type IV collagen in the basement membranes (Kleissl et al., 1978). The fibrils of collagen are bound together in dense bundles that confer on the cervix the rigidity that characterizes its non pregnant and early pregnant condition. A small amount of elastin is also present within the cervix, providing elasticity. There is also a small amount of smooth muscle (<10%) (Thomson & Norman, 2005). The collagen is embedded in a ground substance consisting of large-molecular-weight proteoglycan complexes containing a variety of highly negatively charged repeating disaccharides named glycosaminoglycans (GAGs). The most abundant GAGs are chondroitin and its epimer dermatan sulfate (Uldbjerg et al., 1983; von Maillot et al., 1979). The relationship between the GAG side chains and the collagen fibrils is important in conferring on the cervix its mechanical strength (Lindahl et al., 1978).

Cervical epithelium

The cervix is covered by both columnar and stratified non-keratinising squamous epithelia (Figure 1).

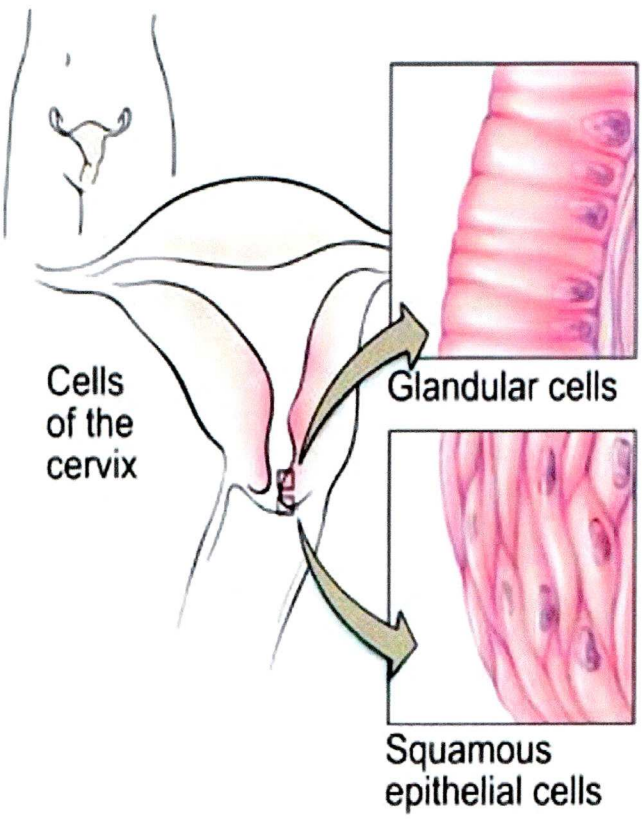


Figure 1: Cervical epithelium
[http://www.cancer-cure-secrets.com/images/cervix3.jpg]

The squamocolumnar junction is where these two meet (Thomas M J in Manual of Clinical Colposcopy,1997). The squamous epithelium of the cervical portion is similar to that of the vagina, except that it is generally smooth and lacks pegs (Figure 2). The "glandular" or columnar epithelium of the cervix, (Figure 3), is located cephalad to the squamo-columnar junction. It covers a variable amount of the ectocervix and lines the endocervical canal. It is comprised of a single layer of mucin secreting cells. The epithelium is thrown into longitudinal folds and invaginations that make up the called endocervical glands (they are not true glands). The squamocolumnar junction

(SCJ) is defined as the junction between the squamous epithelium and the glandular epithelium. Age and hormonal status are the most important factors influencing its location. The SCJ is generally located on the ectocervix at variable distances from the os in reproductive-aged women, as the cervix, and particularly the endocervical canal elongates under the influence of estrogen. The high estrogen levels of pregnancy promote further eversion of the SCJ (Systemic Pathology, 1991).

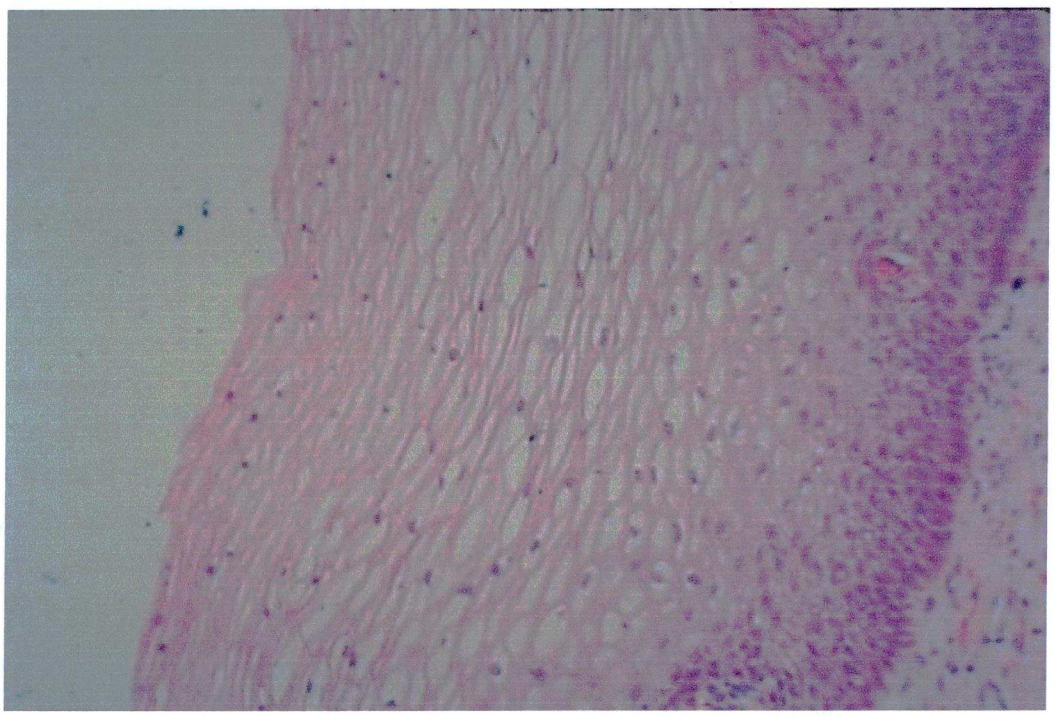


Figure 2: Cervix-Squamous Epithelium
[http://www.pathology.washington.edu/about/education/gallery/jpgs575/spb/img0010.jpg]

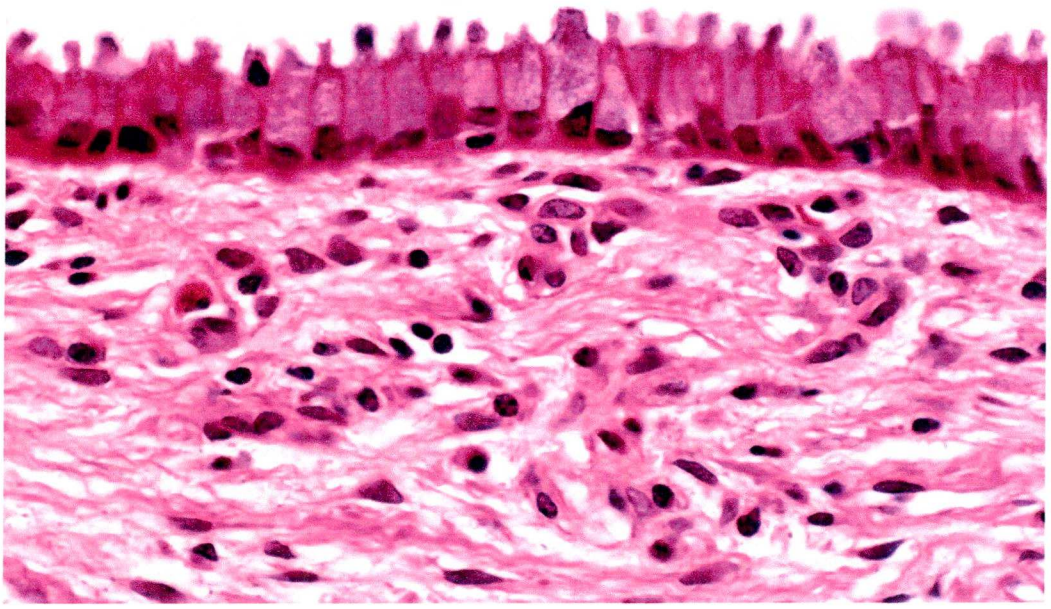


Figure 3: Cervix-Columnar Epithelium
[http://pro.corbis.com/images/42-18705600.jpg?size=67&uid=%7BE29958D9-E639-4DE9-AE98-68A3FE41C63A%7D]

Pregnancy-related changes

The cervix in pregnancy shows stromal oedema, increased vascularity, enlargement of glandular structures, and acute inflammatory response. Stromal decidualization may occur in the second and third trimesters; these changes may appear suspicious to the inexperienced observer (Blaustein's, 1994). For the majority of pregnancy, myometrial contractions need to be repressed, whilst the cervix must remain closed and firm to retain the developing fetus within the uterus. Parturition involves the synchronization of myometrial activity and change in the structure of the cervix, leading to regular co-ordinated uterine contractions and cervical dilatation and effacement.

1.8 Basic principles of Immunity

Immunity protects organisms against foreign micro-organisms, such as bacteria and viruses, and against cancerous cells. The cells that provide this protection are called white blood cells, or leukocytes, and make up the immune system. Leukocytes are colourless, with clear or granulated cytoplasm, and are capable of independent amoeboid movement. They occur in the blood, lymph, and elsewhere in the body's tissues. Unlike mammalian red blood cells, leukocytes possess a nucleus. There are several different types of leukocytes. Some (phagocytes and macrophages) engulf invading micro-organisms, others kill infected cells, while lymphocytes produce more specific immune responses (Janeway et al., 2001).

Phagocytes

Phagocyte is a leukocyte that ingests and destroys foreign matter such as microorganisms or debris by a process known as phagocytosis. Phagocytes are extremely useful as an initial immune system response to infection. Phagocytes contain many lysosomes that enable them to digest foreign material. Phagocytes engulf pathogens, debris, dead or dying cells and extracellular matrix. In addition to engulfing and digesting foreign particles, phagocytes can induce apoptosis of normal and tumor cells, produce cationic proteins, complement components and clotting factors, arachidonic acid metabolites, prostaglandins, leukotrienes, thromboxanes, cytokines, proteases and hydrolases, reactive oxygen and nitrogen intermediates. The main categories of phagocytes are: macrophages (and monocytes) and microphages, such as polymorphonuclear leukocytes (primarily neutrophils)

Neutrophils (Neutrophil granulocytes), are the most abundant type of white blood cells and their name arrives from staining characteristics on hematoxylin and eosin (H&E) histological preparations. Whereas basophilic

cellular components stain dark blue and eosinophilic components stain bright red, neutrophilic components stain a neutral pink (Figure 4).

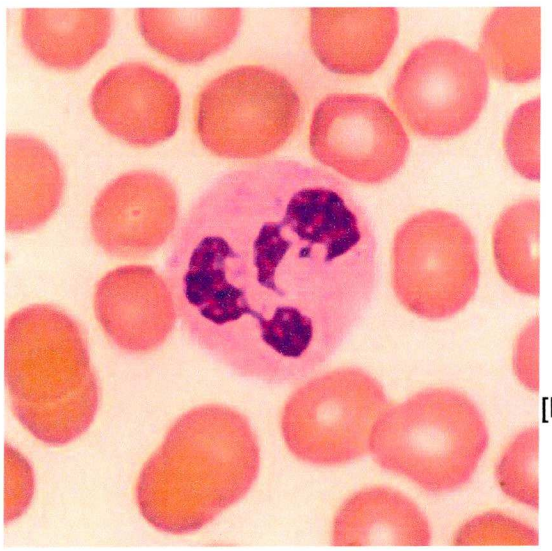


Figure 4 : Neutrophil
[http://www.unomaha.edu/hpa/blood.html]

Neutrophils, being highly motile, undergo a process called chemotaxis that allows them to migrate toward sites of infection or inflammation, attracted by cytokines expressed by activated endothelium, mast cells and macrophages. Neutrophils are phagocytes, capable of ingesting microorganisms or particles. Each phagocytic event resulting in the formation of a phagosome into which reactive oxygen species and hydrolytic enzymes are secreted. Neutrophils react within an hour of tissue injury and are the hallmark of acute inflammation (Cohen et al., 2002).

Monocytes, (Figure 5), are produced by the bone marrow from haematopoietic stem cell precursors called monoblasts. Monocytes circulate in the bloodstream for about one to three days and then typically move into tissues throughout the body. Monocytes which migrate from the bloodstream to other tissues are called macrophages. Macrophages are responsible for protecting tissues from foreign substances (Cohen et al., 2002).

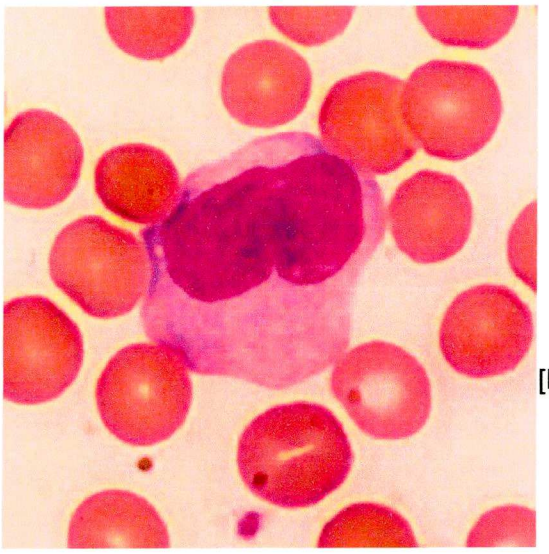


Figure 5 : Monocyte
[http://www.unomaha.edu/hpa/blood.html]

Granulocytes

Granulocytes are a category of white blood cells characterised by the presence of granules in their cytoplasm. They are also called polymorphonuclear leukocytes (PMN or PML) because of the varying shapes of the nucleus, which is usually lobed into three segments. Apart from neutrophil granulocytes, there are two more types of granulocytes, distinguished by their appearance under Wright's stain: Eosinophil granulocytes and Basophil granulocytes.

Eosinophil granulocytes, commonly referred to as eosinophils, are white blood cells of the immune system that are responsible for combating infection by parasites. They also control mechanisms associated with allergy and asthma. Eosinophils develop and mature in bone marrow (Figure 6).

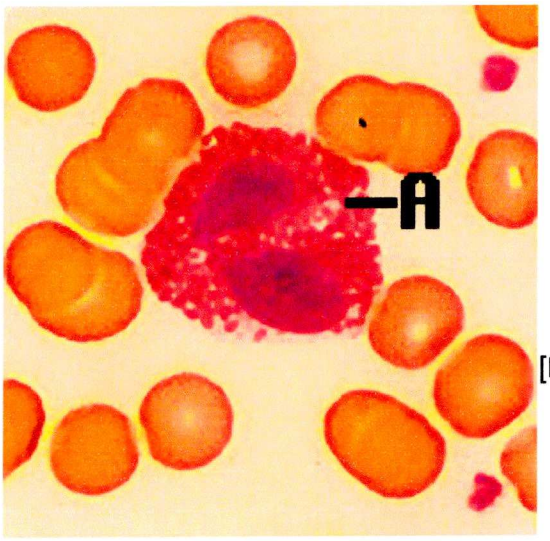


Figure 6 : Eosinophil
[http://www.unomaha.edu/hpa/blood.html]

They differentiate from myeloid precursor cells in response to the cytokines interleukin 3 (IL-3), interleukin 5 (IL-5), and granulocyte macrophage colony-stimulating factor (GM-CSF). After maturation, eosinophils circulate in blood and migrate to inflammatory sites in tissues in response to chemokines (Yamaguchi et al., 1988). At these infectious sites, eosinophils are activated by Type 2 cytokines released from a specific subset of helper T cells. Following activation, eosinophils effector functions include production of: cationic granule proteins; reactive oxygen species such as superoxide; lipid mediators like the eicosanoids and prostaglandin (e.g. PGE_2) families; enzymes such as elastase, growth factors such as TGF beta; cytokines such as IL-1, IL-2, IL-4, IL-5, IL-6, IL-8, IL-13, and TNF alpha (Bandeira-Melo et al., 2002). In addition, eosinophils play a role in fighting viral infections and in fibrin removal during inflammation. Eosinophils are considered the main effector cells in allergic responses and asthma pathogenesis and are associated with disease severity. Eosinophils are also involved in many other biological processes, including postpubertal mammary gland development, oestrus cycling, allograft rejection and neoplasia. Following activation by an immune stimulus, eosinophils degranulate to release an array of cytotoxic granule cationic proteins that are capable of inducing tissue damage and dysfunction (Saito et al., 2004; Horiuchi et al.,1997) .

Basophils are the least common of the granulocytes, representing about 0.01% to 0.3% of circulating leukocytes (Figure 7).

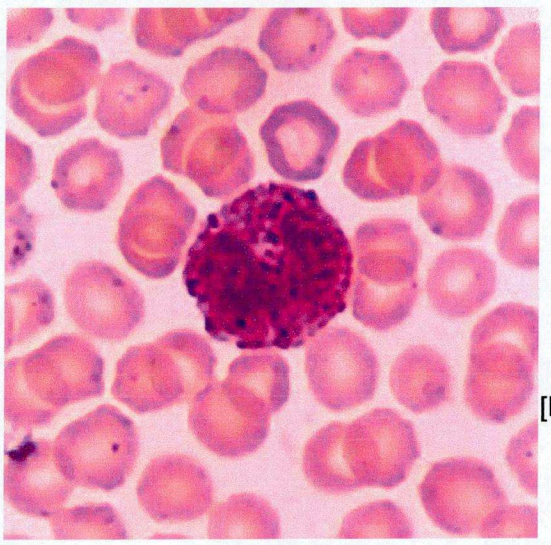


Figure 7: Basophil
[http://www.unomaha.edu/hpa/blood.html]

They contain large cytoplasmic granules and like all circulating granulocytes, basophils can be recruited out of the blood into a tissue when needed. When activated, basophils degranulate to release histamine, proteoglycans (e.g. heparin and chondroitin), and proteolytic enzymes (e.g. elastase and lysophospholipase). They also secrete lipid mediators like leukotrienes, and several cytokines. Each of these substances contributes to inflammation (Janeway et al., 2001).

Lymphocyte

A lymphocyte is a type of white blood cell in the vertebrate immune system (Figure 8). By their appearance under the light microscope, there are two broad categories of lymphocytes, namely the large granular lymphocytes and the small lymphocytes. Functionally distinct subsets of lymphocytes correlate with their appearance. Most, but not all large granular lymphocytes are more commonly known as the natural killer cells (NK cells). The small lymphocytes are the T cells and B cells.

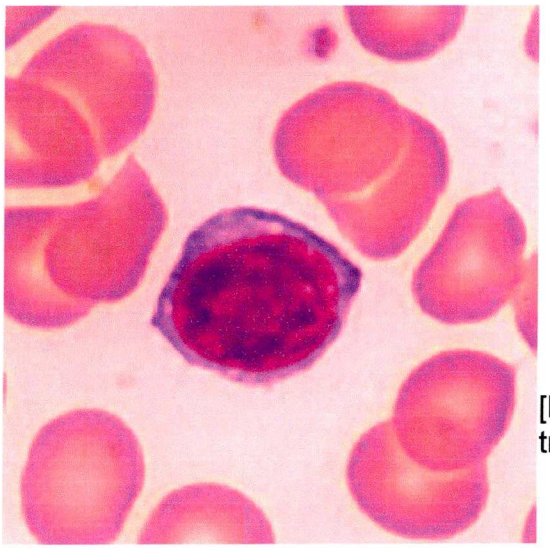


Figure 8: Lymphocyte
[http://www.unomaha.edu/hpa/blood.html]

T cells belong play a central role in cell-mediated immunity. They can be distinguished from other lymphocyte types, such as B cells and NK cells by the presence of a special receptor on their cell surface that is called the T cell receptor (Schwarz et al., 2006).

Several different subsets of T cells have been described, each with a distinct function.

- Helper T cells, which once activated, they divide rapidly and secrete small proteins called cytokines that regulate or "help" the immune response.
- Cytotoxic T cells (T_c cells) destroy virally infected cells and tumor cells, and are also implicated in transplant rejection. These cells are also known as $CD8^+$ T cells, since they express the CD8 glycoprotein at their surface.
- Memory T cells are a subset of antigen-specific T cells that persist long-term after an infection has resolved. Memory cells may be either CD4+ or CD8+.
- Regulatory T cells (T_{reg} cells), formerly known as suppressor T cells, are crucial for the maintenance of immunological tolerance. Their major role is to shut down T cell mediated immunity towards the end of an immune reaction and to suppress auto-reactive T cells that escaped the process of negative selection in the thymus. Two major classes of CD4+ regulatory T cells have been described, including the naturally occurring T_{reg} cells and the adaptive T_{reg} cells.
- Natural Killer T cells (NKT cells) are a special kind of lymphocyte that bridges the adaptive immune system with the innate immune system. Once activated, these cells can perform functions ascribed to both T_h and T_c cells (i.e. cytokine production and release of cytolytic/cell killing molecules).
- γδ T cells represent a small subset of T cells that possess a distinct TCR on their surface (Schwarz et al., 2006).

B cells are lymphocytes that play a large role in the humoral immune response as opposed to the cell-mediated immune response that is governed by T cells. The principal function of B cells is to make antibodies against soluble antigens. B cells are essential component of the adaptive immune system. They do not produce antibodies until they become fully activated. Each B cell has a unique receptor protein, referred to as the B cell receptor, on its surface that will bind to one particular antigen (Li et al., 2006).

Several different subsets of B cells have been described, each with a distinct function.

- Plasma B cells (also known as plasma cells) are large B cells that have been exposed to antigen and are producing and secreting large amounts of antibodies, which assist in the destruction of microbes by binding to them and making them easier targets for phagocytes and activation of the complement system.
- Memory B cells are formed from activated B cells that are specific to the antigen encountered during the primary immune response. These cells are able to live for a long time, and can respond quickly following a second exposure to the same antigen.
- B-1 cells express IgM in greater quantities than IgG and its receptors show polyspecificity, meaning that they have low affinities for many different antigens, but have a preference for other immunoglobulins, self antigens and common bacterial polysaccharides (Li et al., 2006).

Natural killer cells (or NK cells) are a type of cytotoxic lymphocyte, which constitute a major component of the innate immune system. NK cells play a major role in the rejection of tumors and cells infected by viruses. The cells kill by releasing small cytoplasmic granules of proteins called perforin and granzyme that cause the target cell to die by apoptosis (Oldham, 1983). NK-cells are defined as large granular lymphocytes, which usually express the surface markers CD16 and CD56. NK cells are activated in response to interferons or macrophage-derived cytokines. They serve to contain viral infections while the adaptive immune response is generating antigen-specific

cytotoxic T cells that can clear the infection. To control their cytotoxic activity, NK cells possess two types of surface receptors: "activating receptors" and "inhibitory receptors". These inhibitory receptors recognize MHC class I alleles (Oldham, 1983).

Cellular immunity

Historically, the immune system was separated into two branches: humoral immunity, for which the protective function of immunization could be found in the humor (cell-free bodily fluid or serum) and cellular immunity, for which the protective function of immunization was associated with cells. Thus, cell-mediated immunity is an immune response that does not involve antibodies but rather involves the activation of macrophages, natural killer cells (NK), antigen-specific cytotoxic T-lymphocytes, and the release of various cytokines in response to an antigen.

Cellular immunity protects the body by:

- activating antigen-specific cytotoxic T-lymphocytes that are able to induce apoptosis in body cells displaying epitopes of foreign antigen on their surface, such as virus-infected cells, cells with intracellular bacteria, and cancer cells displaying tumor antigens;
- activating macrophages and natural killer cells, enabling them to destroy intracellular pathogens; and
- stimulating cells to secrete a variety of cytokines that influence the function of other cells involved in adaptive immune responses and innate immune responses (Gary 2007).

1.9 Cervical ripening: an inflammatory-like reaction

Prior to the onset of labour the cervix undergoes a process known as ripening during which it becomes softer, and shorter with a decrease in its concentration and an increase in water content (Olah and Gee 1992). This process allows the cervix to be pulled open passively by uterine contractions during parturition. Extensive remodelling of connective tissue plays an important physiological role in the cervical ripening (Gabor et al.,1991). Connective tissue changes during cervical ripening include many components similar to an inflammatory reaction (Liggins 1981). The biochemical changes accompanying cervical ripening include a decrease in collagen concentration, due to both a decline in collagen synthesis and increased collagenase activity (Westergren-Thorsson et al., 1998, Iwahashi et al., 2003). In vivo studies have shown decreased cervical collagen organisation and content in the third trimester of pregnancy as labour approaches (Maul et al., 2003). Cervical ripening is induced by PGE2 (O'Brien et al., 1995) whereas degradation of collagen is promoted by IL-1, platelet-activating factor, and mechanical stretch (Terakawa et al., 2002; Ou et al., 2000; Winkler et al., 2001). The effects of PG on cervical dilatation in primate parturition may be modulated through relative expression of prostanoid receptor subtypes (Smith et al., 2001). In addition, local metabolism of progesterone by 5-α reductase is required for cervical remodeling and successful delivery (Mahendroo et al., 1999; Minjarez et al., 2001).

There is additional support that cervical ripening is associated with an increase in cervical prostaglandin, proinflammatory cytokines, cell adhesion molecule and nitric oxide synthase production (Ellwood et al., 1980, Ledingham et al, 2000) and a decrease in prostaglandin breakdown (Tornblom et al., 2004). Some, but not all, of these events occur in concert with cervical leukocyte invasion (Osman et al., 2002), and many of these agents can be co-localised to cervical leukocytes using immunohistochemistry (Young et al., 2002). Leukocytes are thought to migrate into the cervix before labour (Luo, Ibaragi et al. 2000; Osman, Young et al. 2003). This cervical

leukocyte infiltrate is composed principally of granulocytes and macrophages (Osman, Young et al. 2003) which are then though to secret inflammatory cytokines (Luo, Ibaragi et al. 2000; Winkler 2003) which in turn initiate labour (Kurkinen-Raty, Ruokonen et al. 2001; Romero, Espinoza et al. 2002; Keelan, Blumenstein et al. 2003; Kishida, Yamada et al. 2003). However, previous investigators have studied the cervix after delivery not immediately prior to the onset labour (Yoshimura et al. 1987; Knudsen et al. 1997; Luo et al. 2000; Terzidou and Bennett 2002; Osman et al. 2003; Sakamoto et al. 2005), or found a much greater increase in cervical leukocytes postpartum (Bokstrom et al. 1997). Thus, the cervical lymphocyte infiltrate previously described could be an effect rather than a cause of labour. Furthermore a mouse model of preterm labour found the leukocyte infiltrate only occurred during and after labour not prior to the onset of labour (Timmons and Mahendroo 2006). Hence, the question remains as to whether cervical leucocyte infiltration is a causative factor in initiating preterm labour.

Cervical leukocytes populations have been found to be different in the stromal and sub-epithelial compartments of the cervix (Prakash, Patterson et al. 2001; Sakamoto, Moran et al. 2005).

Data on cervical changes during preterm labour are more limited, although a recent series of elegant studies from Sweden have shown that preterm parturition is associated with an increase in interleukin (IL)-6 and -8 and monocyte chemotactic protein-1 concentrations, and a decrease in prostaglandin dehydrogenase, even in the absence of infection (Tornblom SA, Klimaviciute A et al., 2005). Furthermore, preterm labour was associated with greater levels of each of the NOS isoforms than term labour, with eNOS expression in preterm labour greater than in non-labouring gestation-matched controls (Tornblom SA, Maul H et al., 2005).

Taken together, these data imply that similar processes occur in term and preterm cervical ripening. Once ripened, further cervical dilation is induced by contractions of the myometrium.

Recent advances in potential bio-psychosocial causal pathways to understand the underlying mechanisms responsible for early cervical modifications and preterm uterine activity have renewed interest and enthusiasm to study these mechanisms. A transdisciplinary approach has already brought newer insights, such as inheritance susceptibility, the role of inflammatory processes rather than infection, and the maternal or fetal activation of the hypothalamic–pituitary–adrenal axis, which antedate the initiation of preterm parturition.

Aims of this thesis:

1. To describe cervical epithelial leukocyte population in women with a past history of idiopathic preterm labour

2. To investigate how the cervical epithelial leukocyte population change in pregnancy

3. To investigate the hypothesis that a cervical leukocytosis was a prelude to cervical shortening and funnelling.

4. To investigate the hypothesis that recurrent preterm labour is associated with a different cervical leukocyte population

5. To investigate the cervical leukocyte response to abnormal genital tract pathogens.

Chapter 2

Cervical epithelial leukocyte population in women with a past history of idiopathic preterm labour

Chapter 2

Cervical epithelial leukocyte population in women with a past history of idiopathic preterm labour

2.1 Introduction

2.1.1 The cervical mucus

Human cervical mucus is a highly hydrated viscoelastic gel composed of a group of extraordinarily long, glycosylated, highly hydrated proteins known as mucins. It is secreted by the endocervical glands of the uterine cervix, called cervical crypts. These crypts run the entire length of the cervix with 20-50 folds totalling some 200-900 crypts (Figure 9). Cervical mucus is located between microbe-rich vagina and the normally sterile uterine cavity, which suggests a host defence function other than a physical barrier preventing ascending infections (Hein et al., 2001).

The physical, chemical, rheological, and hydrodynamic properties of the mucus change dramatically during the ovulatory period (Lagow et al., 1972). Immediately before ovulation, high estrogen levels stimulate secretion of a large amount of thin, watery mucus that is hospitable to sperm transit. The stringy, elastic quality of cervical mucus during the ovulatory period allows it to be formed into a long thread. This type of mucus is called E mucus, because it is produced by type E cervical crypts. It is further divided into L mucus for 'locking in' because of its ability to act as a filter to imperfect or malformed sperm and S mucus for 'sperm transmission'. After ovulation, high progesterone levels stimulate secretion of thick, viscous mucus that is less hospitable to sperm. This cervical mucus is produced by type G cervical crypts, thus is characterised as G mucus. It acts as a natural barrier to sperm and does not facilitate transmission through the genital tract, while the

presence of leukocytes and immunoglobulins imply that the G mucus is part of the immune system, which protects the woman's reproductive system from infection. Further microscopic studies (Odeblad 1994) revealed that the G mucus always contains varying numbers of leukocytes and immunoglobulins usually more copious after ovulation that before it.

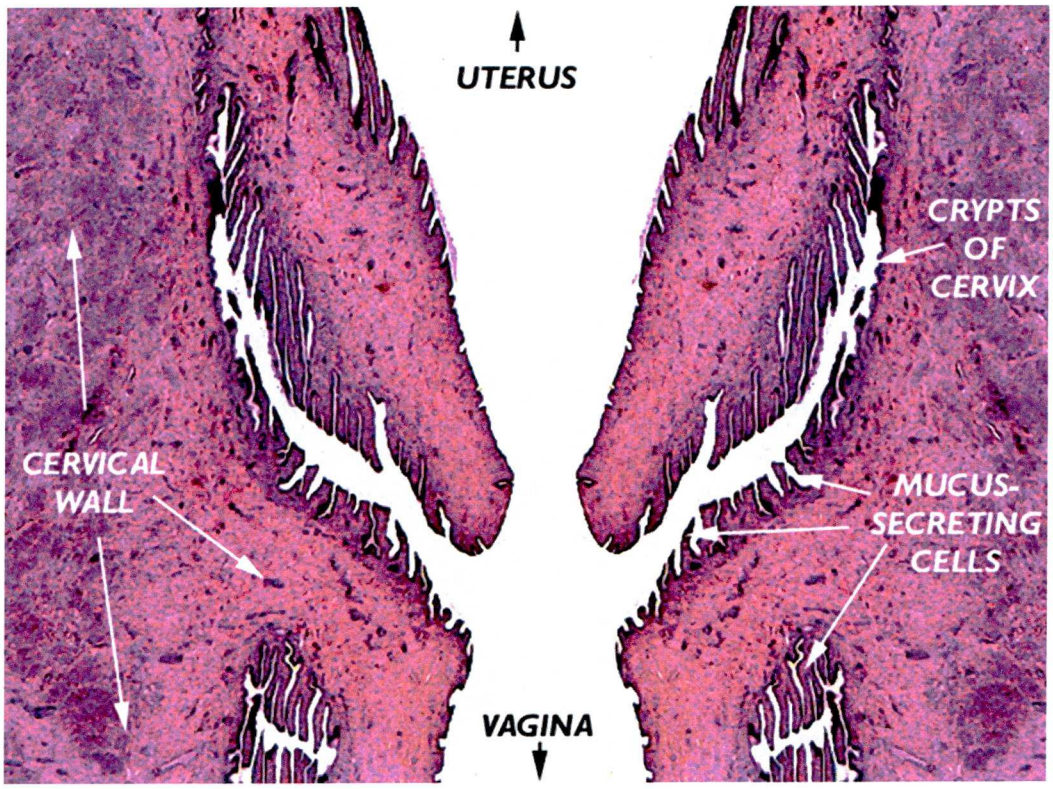


Figure 9: Cervical crypts and mucus secreting cells
[http://education.vetmed.vt.edu/curriculum/vm8054/labs/Lab28/IMAGES/LS%20THROUGH%20CERVIX%20CHANNEL%20SMALL.JPG]

After conception, cervical mucus undergoes a dramatic change under the influence of progesterone, which modifies both its appearance and physicochemical properties. The cervical mucus becomes thick, sticky, viscous, opaque, and gelatinous and forms a plug that obstructs the cervical canal. At vaginal inspection, the plug appears in the cervical os as a clot of dense mucoid material (Moghissi et al., 1972; Wolf et al., 1978). The cervical mucus plug is considered to be a physical barrier that prevents ascending infection by microorganisms located in the genital tract.

2.1.2 Structure and Biochemistry of the Cervical Mucus

Two different fragments consist the entirety of the cervical mucus: The low viscosity component (aqueous phase) and the high viscosity component (the insoluble gel phase) (Schumacher et al.,1970). The aqueous phase contains soluble components which are not only supplied by the cervical mucosa but some are derived from the peritoneum, ovaries, fallopian tubes, vagina and by transudation from serum (Odeblad 1968).

The major components of the aqueous phase of cervical mucus are lipids (Singh and Swartoot 1972), and more particular, triglycerides, phospholipids, cholesterol and cholesterol esters. Their amount alters due to the hormonal changes in the menstrual cycle (Singh and Swartoot 1972).

Traces of potassium, magnesium, calcium, copper, zinc, phosphate, sulphate and bicarbonate can also be detected. Evidently, it has been demonstrated that an excess of one trace element can exhibit a relative deficiency of another (Daunter et al., 1977).

Low levels of secretory immunoglobulin A (IgA) and G (IgG) constitute the aqueous phase components of the cervical mucus (Coughlan et al., 1977). IgA is the first line of defence against microbes entering through mucosal surfaces and IgG is the most abundant immunoglobulin in the blood and the only antibody to cross the placenta to provide passive humoral immunity to the fetus. Thus, both antibodies are vital in combating various infections in the cervix.

Additionally, a number of proteins have been recognized in human cervical mucus (Treves et al., 1986) such as lysozyme, alkaline phosphatase and lactoferin. Lysozyme is an enzymatic protein with action against some bacterial cell wall structures (Glynn et al., 1967). Alkaline phosphatase has been confirmed to be consistently low under the influence of oestrogens and high after administration of gestagens (Smith et al., 1977). Lactoferin is a

protein that prevents the growth of pathogens, controls cell and tissue damage caused by oxidation and facilitates iron transport. It is present in a number of human secretions such as cervical mucus, semen, urine, saliva, nasal secretions, tears and pancreatic fluid (Masson et al., 1966).

The insoluble gel phase of the cervical mucus is constituted mainly from a thread-like glycoprotein called mucin (Schumacher et al., 1970). Glycoproteins are essential components of cell membranes and take part in the manufacture of connective tissues such as collagen. They also have lubricative properties and are used as protective agents. Glycoproteins consist of a polypeptide covalently bound to a carbohydrate moiety. The saccharide chains, referred to as glycans, can be linked to the polypeptide in two major ways (Carlstedt et al., 1989). N-linked glycans are attached through N-acetylglucosamine to the amide nitrogen of an asparagines molecule. N-acetylglucosamine is simply a glucose molecule, which is bound to an amine group. O-linked glycans consist of N-acetylgalactosamine attached to the O-terminus of a threonine or serine residue. N-acetylgalactosamine is simply a galactose molecule with an amine group covalently bound to the second carbon.

Cervical mucin contains many short O-linked glycans. These glycans are characterized by their high proportion of carbohydrate, more than 40% distributed along the peptide core in the form of numerous side chains. The oligosaccharide side chains of the glycoprotein that make up the mucin may terminate with either L-fucose or sialic acid(s). The glycoproteins of cervical mucin also bind water mainly by hydrogen bonding and form a hydrogel (Carlstedt et al., 1989).

Besides cervical mucin, the insoluble gel phase of cervical mucus contains a variety of mixed population of leucocytes (Prakash M, Kapembwa M, et al., 2001). Leucocytes are involved in helping the body to combat infection and isolate dead tissue, foreign bodies and bacteria. Thus, their presence in the cervical mucus could have a key role on cervical ripening.

In theory, a cervical mucus sample properly taken and successfully disrupted contains a variety of cells, some of which have been exfoliated locally and trapped within the mucus and others by the region of the external os. A cervical mucus sample therefore may contain:

- Leukocytes
- Cells from the original squamous epithelium of the cervix
- Cells from the colulmnar epithelium of the endocervical canal
- Cells from the metaplastic epithelium of the transformation zone
- Cells from other parts of the genital tract, e.g. endometrial cells
- Commensal organisms

Studies of the role of cytokines in parturition suggest that analysis of leukocyte populations in the cervix at the time of parturition may be worth-while, although technically difficult.

Prakash M., S. Patterson et al. 2001 described the cervical mucous population in the non-pregnant state. There are no previously published studies of cervical mucus leukocytes in pregnancy. Hence, the aim of this part of the project was to describe the cervical leukocyte population in women with a past history of idiopathic preterm labour.

2.1.3 Comparison of the Specimen Collection Techniques

Several methods have been described for collecting material from the cervix for cytological studies, each of which has its advantages and disadvantages. Three of the most widely used are:

1. The use of a cotton swab to obtain cervical mucus cells. With this technique, rotating a cotton swab 360° in the external os of the cervix, cervical mucus cells could be obtained. Although this procedure is not invasive, a small number of cells per sample are obtained.

2. The cervical aspiration using an aspirette which is a symmetrical tube, (diameter approximately 3mm, graded in I cm increments) with a Teflon plunger (Figure 10). When the aspirette is inserted 2cm into the cervical os, the plunger is withdrawn and the cervical mucus is collected. Again, this technique is not invasive, but is not the most recommended. That is because only the mucin matrix with no endocervical cells can be extracted.

3. The cervical scrape using a cytobrush. The cytobrush is a small brush with nylon bristles (Figure 11). It collects an abundant endocervical cell material also reaching into the endocervical crypts. Cytobrush has been known to be an invasive, non-traumatic method. Although there were some minor problems with the samples obtained by cytobrush, it is strongly recommended as the instrument used for all specimen collection, collecting on average a larger number of leukocytes and a large number of epithelial cells as it scrapes the mucosa layers of the intraepithelial wall.

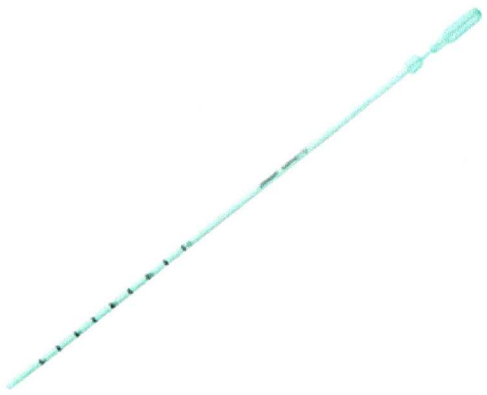


Figure 10: Aspirette
[http://www.coopersurgical.com/PublishingImages/8700-Med.jpg]

In order to describe cervical epithelial leukocyte population in women with a past history of idiopathic preterm labour, we adapted a previously described, non-traumatic method of sampling the cervix which Prakash M., S. Patterson et al. (2001) have described to sample the non-pregnant cervix.

Figure 11: Cytobrush
[http://shop.doccheck.com/de/out/1/html/0/dyn_images/1/cryobrush_152x152_p1.jpg]

2.2 Methods

2.2.1 Subjects for the main study

The study was approved by the Liverpool Adult Local Research Ethics Committee. One hundred and six women were recruited from an antenatal clinic dedicated to the care of women with a history of preterm labour at the Liverpool Women's NHS Foundation Trust. All women gave written consent before enrolment in the study.

The inclusion criterion was at least one prior spontaneous birth between 22 and 32 weeks of gestation. In Liverpool Women's Hospital, preterm deliveries with positive microbiology or inflammation detected on placental histology are considered to be idiopathic as the clinician can never be sure whether the infection came before or after the onset of labour. For this reason, women whose preterm birth was thought to be contributed to by infection or inflammation were included in the study. We did not include women who experienced preterm labour but did not deliver prior to 32 weeks gestation. Women were excluded from the study if they had a multiple pregnancy or if their previous preterm labour had a recognisable cause, e.g. placental abruption, intrauterine growth restriction or pre-eclampsia, or was iatrogenic. Women were also excluded if they had had a previous cone biopsy. Women were not included if they had received antibiotics in pregnancy prior to their appointment. As suggested by Prakash M., S. Patterson et al. (2001), cervical samples were excluded if contaminated by blood (= 10). Samples were also excluded from this part of our study if a pathogenic genital tract infection was detected on the swabs or if they had bacterial vaginosis and were subsequently given antibiotics (n = 15). We used these samples to investigate the cervical leukocyte response to abnormal genital tract pathogens (chapter 6).

As result, eighty-one women were finally included in the first part of our study and had their cervical leukocyte populations sampled at 12–16 weeks of gestation.

Information regarding age, race, obstetric history, complications during the current pregnancy and sociodemographic factors of the patient was obtained from the medical records and by a standardised questionnaire. The questionnaire was distributed and explained during the first study visit. All women had sufficient education to read the patient information leaflet and sign their name on the consent form. The median age was 30.6 (range 48–19) years. 66 were Caucasian, 5 Asian and 10 Afro-Caribbean. Body mass index was calculated with the formula (Weight [kg]/Height2 [m]). The weight used in this calculation was the reported pre-pregnancy weight recorded during the woman's first prenatal visit. The median BMI was 25.3 (range 21-45). Fifteen of these women had a previous ectopic pregnancy and five had two previous ectopic pregnancies. Twenty one women had a previous miscarriage and nine had two previous miscarriages. Twelve women had a previous term delivery and six had two previous term deliveries (Table 2.1).

Gestational age was based upon transvaginal ultrasonographic measurements and recorded in completed weeks and days.

Table 2.1. Summary of patient characteristics at study entry (n=81)

Age (range)	30.6 (48-19)
BMI (range)	25.3 (45-21)
Ethnicity	
Caucasian	66
Afro-Caribbean	10
Asian	5
Number of women with previous ectopic pregnancies	
one previous ectopic pregnancy	15
two previous ectopic pregnancies	5
Number of women with previous early miscarriages	
one previous early miscarriage	21
two previous early miscarriages	9

Number of women with previous term deliveries	
one previous term delivery	12
two previous term deliveries	6

2.2.2 Sample collection

The subjects were placed in a supine position with feet placed in stirrups (the standard position for gynaecological exanimation). A plastic speculum was inserted into the vaginal canal, exposing the external os. Cervical mucus was then obtained using a fine cervical cytobrush (Cervex-brush, Rovers Medical Devices, Oss, Netherlands). It was important that a few bristles of the brush were seen outside the cervix. Then the cytobrush was rotated slowly one turn and withdrawn (Figure 12).

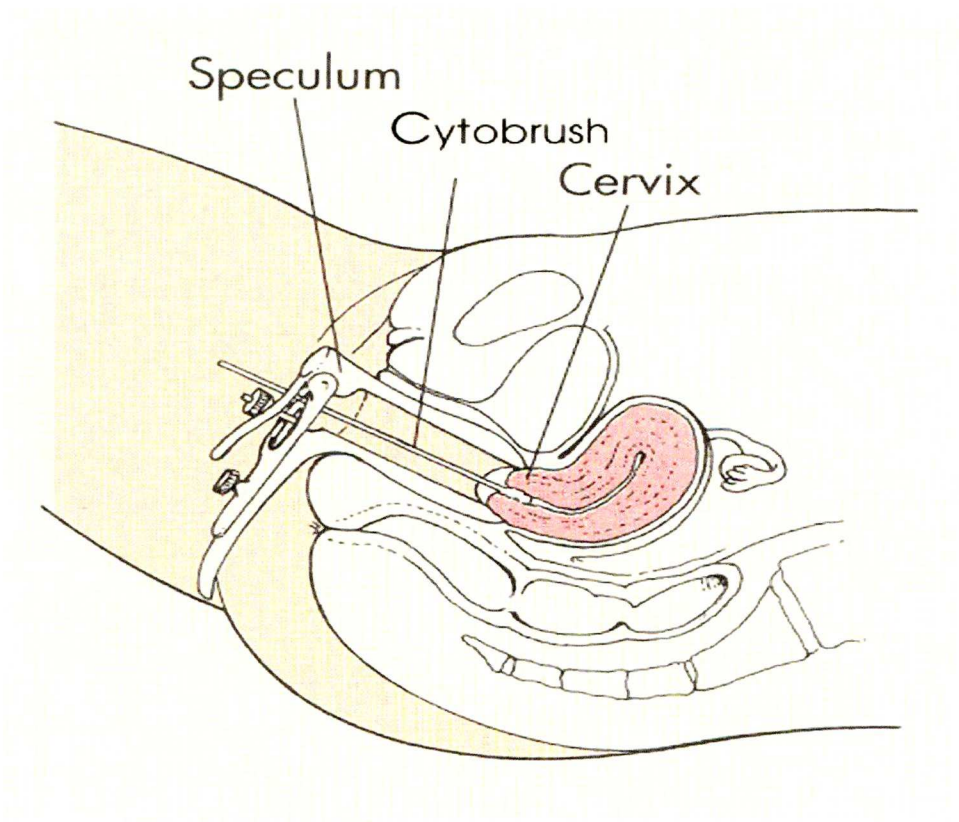


Figure 12: Procedure of cervical mucus collection
[http://cal.fmc.flinders.edu.au/gemp/ClinicalSkills/clinskil/year2/pelvic/graphics/mosby1721.jpg]

2.2.3 Sample processing

Samples were immediately diluted in 5 ml of phosphate-buffered saline (PBS) containing 0.1% bovine serum albumin (BSA). Samples were mixed well to disrupt the mucus. As cervical mucus is a vicious gel-like fluid, solubilisation is necessary to ensure a homogeneous mixture. Sonication was proved to be an efficient method for dispersion of cervical mucus (Eggert-Kruse et al., 2000). For this reason, the samples were centrifuged for 10 min at 1200 rpm at room temperature. The pellet was then snap frozen and stored at −80 °C. For use the pellet was re-suspended in 2 ml PBS containing 0.1% BSA and diluted 1:1 in trypan blue for cell counting. Cell number was determined using a haemocytometer (Weber Scientific, Hamilton, USA). All cells were counted including epithelial cells. If it was deemed necessary the cells were diluted in PBS-BSA to give a concentration of $1x10^6$ / ml. If the samples were already less than $1x1O^6$ / ml no dilution was done.

2.2.4 Immunohistochemistry

The APAAP immunoalkaline phosphatase technique was used to demonstrate the antigens present in the mucus samples in situ by the use of specific antigen-antibody interactions (Appendix 1-3). A two steps indirect method was used for labelling the antibodies rather than a direct method, which lacks flexibility. The indirect method utilises a pre-formed, cyclic enzyme anti-enzyme immune complex APAAP (alkaline phosphate-anti-alkaline phosphatase). This complex comprises of three enzyme molecules and two antibody molecules and uses the alkaline phosphatase enzyme and antibodies directed against it. The APAAP used was mouse monoclonal. The primary antibodies used were mouse monoclonal antibodies with rabbit anti-mouse immunoglobulin being the secondary antibody used. In table 2.2 we can see the entirety of the antibodies that we used in our study. We did not examine the subpopulation of the CD2- Memory T cells, CD6-Regulatory T

cells (formerly known as suppressor T cells), CD11- γδ T cells, CD19-Memory B cells and CD79-Plasma cells due to financial reasons.

Table 2.2. Primary monoclonal antibodies

Monoclonal antibody	Specificity	Source	Clone	Dilution
CD45	All haematopoetic cells	Culture supernatant, American Type Culture Collection	F10894	Neat
CD3	T lymphocytes	Serotec	MCA 463	1:100
CD4	T (helper) cells	Dako	M0716	1:50
CD8	T(cytotoxic) cells	Dako	M0707	1:50
CD14	Macrophages	Culture supernatant, American Type Culture Collection	3C10	Neat
CD16	Natural killer cells, T subset, macrophages, granulocytes	Pharmingen	30621A	1:100
CD20	B lymphocytes	Immunotech	1925	1:50
CD56	Natural killer cells, T subset	Serotec	MCA591	1:50
CD69	Activated T, B, NK cells and macrophages	Serotec	MCA1442	1:100
Mouse IgG	Negative control	Serotec	MCA928	1:100

Normal Human Serum is used in conjunction with the secondary antibody rabbit anti-mouse immunoglobulin, to prevent non-specific binding or blocking the complex protein. Alkaline phosphatase acts in the enzyme substrate reaction to convert the chromatogen (Fast Red TR salt) into a

coloured precipitate based on a reduction of tetrazolium salts. 10mM levamisole is included in the substrate solution to quench and therefore prevent any endogenous alkaline phosphatase activity. This would result in the deposition of a false marker due to the conversion of the phosphatase substrate and produce background staining thus making true positive staining difficult to identify. Twelve mouse monoclonal antibodies (Table 1) were used to assess specific leukocyte populations. Mouse IgG was used as a negative control for the cervical mucus as it should not react with human cell surface antigens.

After removal of the pre-prepared slides from the freezer to allow them to reach room temperature, the areas to be stained were marked with a Dako pen. The slides were fixed in acetone for 10 minutes. The acetone had been placed in the freezer for 10 minutes prior to use to allow it to cool. Cells were rehydrated in 0.05 M Tris-buffered 0.15 M saline (TBS), pH 7.6 for 10 min. The slides were placed in a humidified chamber to avoid them drying out whilst the primary antibodies were diluted in TBS and 0.5% BSA (Sigma). The primary antibodies used were mouse monoclonal antibodies.50µl per spot was added and incubated for 30 minutes in a humidified chamber, at room temperature. After rinsing in TBS, the slides were washed twice in TBS, each wash lasting 5 minutes.

The secondary antibody rabbit anti-mouse immunoglobulin (Dako Z-259) was diluted to a 1:25 concentration in TBS and 5% Normal Human Serum (NHS) (Sigma). 50µl per spot was added and incubated in a humidified chamber for 30 minutes, at room temperature. The slides were then rinsed in TBS and then washed twice in TBS; each lasting for 5 minutes.

The APAAP complex (Serotec STAR 67) was diluted to a 1:50 concentration in TBS and 50µl per spot was added. The slides were incubated in a humidified chamber for 30 minutes at room temperature. The slides were then rinsed in TBS and then washed twice in TBS; each wash lasting 5 minutes.

Bound primary mAbs were detected by incubation with Fast Red (Sigma, Dorset, UK). 10 mg Fast Red TR salt was weighed out into a glass bottle and 10ml of alkaline phosphatase substrate was added. Using 0.45µm filter paper, the solution was filtered into a second glass bottle.

50µl per spot was added and incubated for 20 minutes, at room temperature, in a humidified chamber. The slides were then rinsed once with TBS and then once with distilled water and subsequently counterstained with Haemalum blue (VWR international) for 30 seconds. The slides were then rinsed thoroughly with tap water, and allowed to air dry at room temperature. When completely dry the slides were mounted using Aquamount solution (BDH, Poole, UK).

All the timings of the procedure were exact and timed using a stop clock. It is essential throughout all staining that the slides are not allowed to dry out. In order to avoid this, the slides were placed in an appropriate buffer and during incubation were placed in a humidified chamber.

2.2.5 Quantification of cells

All cells were counted in 10 fields of 400× magnification using a 10 mm × 10 mm graticule covering an area of 0.0625 mm2 by an observer blind to the origin of the sample (Appendix 4 & 5). The positive cells observed were stained red and the negative cells stained blue. In all the CD45 stained samples, the positive and negatively stained cells on each spot were counted in relation to each other. Thus, producing a total number of cells per 10 high powered fields and allowing subsequent counts to be expressed as a percentage of the total number of cells. For the patient samples, which contained insufficient cell numbers in the cervical mucus, the entire spot for each stain was counted instead of 10 fields. A clicker counter was used to ensure precise counting of the cells. In the IgG negative control count, the number of positively stained cells was counted per field to ensure that specific staining was obtained in all other antibody staining.

2.2.6 Data and statistical analysis

Statistical analysis was performed with Stats Direct software (Cheshire, UK). We analysed the mean of CD45+ cells [total leukocytes] as a fraction of total dispersed cells.

2.3. Results

First of all, using the cytobrush technique, a similar proportion of the total cell population including epithelial cells were leukocytes compared to that reported in the previous publication, confirming our ability to reproduce the technique (Prakash M, Kapembwa M et al., 2001). There are data to suggest that leukocytes obtained by the cervical cytobrush are mainly derived from the intraepithelial compartment and that it is possible to obtain samples without significant blood leukocyte contamination (Prakash M, Kapembwa M et al., 2001). Possible blood contamination could lead to erroneous interpretations, as suggested by Prakash M, Kapembwa M et al. (2001). For this reason, samples were excluded from the beginning if contaminated by blood (n = 10).

Analysing our results, leukocytes were found in every sample tested, using an antibody against antigen CD 45 present on all leukocytes. The mean of total leukocytes as percentage of total dispersed cells was 17.4 %. After the incubation with the appropriate primary antibody, we could evaluate the subpopulations of the leukocytes in every sample.

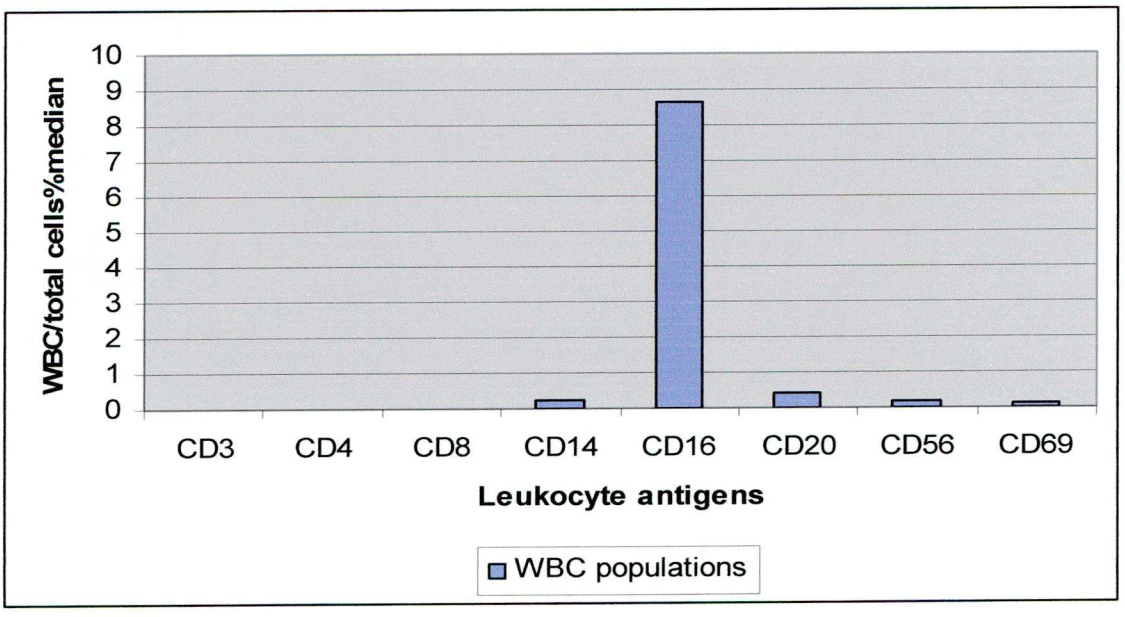
The most common leukocytes present were positive for CD16 (8.66 % of all the cells) (Figure 13). No staining observed in negative controls with mouse IgG (Figure 14). Antibodies to CD16 stain both granulocytes and NK cells. As antibodies to CD56 also stain NK cells and, because there were minimal number of CD56+ cells, we can assume that anti-CD16 stained granulocytes. Thus, the dominant leukocyte detected in this study was the granulocyte. The second most prevalent leukocyte was CD20+ B cells (0.42 % of all cells). CD14 positive macrophages were detected in some samples

(0.24 % of all cells). There were very few T cells. CD56+ were the 0.17% of all the cells (Table 2.3).

Table 2.3. Leukocyte populations (n=81)

Leukocyte antigen	Percentage leukocytes/total cells median (inter quartile range)
CD45	17.4 (3.2-26.8)
CD3	0 (0-0.30)
CD4	0 (0-0.25)
CD8	0 (0-0.18)
CD14	0.24 (0-0.34)
CD16	8.66 (0.1-14.6)
CD20	0.42 (0.09-4.5)
CD56	0.17(0-3.4)
CD69	0.1(0-0.3)

Chart 1: Leukocyte populations



2.4 Discussion

Previous studies using specimens from hysterectomy have demonstrated that leukocytes are present throughout the reproductive tract of women, including the fallopian tubes, uterine endometrium, endocervix, ectocervix, and vagina (Givan et al., 1997).

Our study has comprehensively characterised cervical epithelial leukocytes sub-populations in pregnant women. Using the cytobrush we sampled the intraepithelial layer of the cervix 106 pregnant women with history of previous preterm delivery. We are not aware of any other study on evaluation of the leukocytes population in pregnant women with history of previous preterm labour.

Prakash M, Kapembwa M et al.(2001), evaluated the leukocytes sub-populations both in cervical mucus and peripheral blood specimen in thirteen asyptomatic woman and their data suggests that leukocytes obtained by the cervical cytobrush are mainly derived from the intraepithelial compartment and that it is possible to obtain samples without significant blood leucocyte contamination. In our study, 10 women were finally excluded due to their sample contamination by blood. Our immunohistochemical study showed that the population of leukocytes compared to epithelium cells, varied considerably. Leukocytes were found in every sample tested, using an antibody against antigen CD 45 present on all leukocytes. In agreement with our observation, older studies in non pregnant women suggest that leukocytes are present in cervical tissues of all women regardless of the presence of demonstrable infection and regardless of the diagnosis of cervicitis (Judy et al., 1998). We also found the most prevalent cervical epithelial leukocyte was the CD16+ granulocyte. Granulocytes were not examined by Prakash et al. (2001a) in the non-pregnant cervix, but they were the most prevalent leukocyte sub-type in non-pregnant, non-infected women in our unit

(unpublished data). Sakamoto et al., (2005) taking cervical biopsies from twenty six pregnant women, found a low number of granulocytes in the sub-epithelial and stromal regions. Bokstrom et al., (1997) had the same result when they studied cervical biopsies taken from twenty four pregnant women. However, to date, no investigators have examined epithelial granulocyte expression. We propose that granulocytes are actively secreted into the cervical mucus due to our finding of high numbers of granulocytes in the epithelial cervical region. This is supported by the finding of high numbers of granulocytes in the cervical mucus of both pregnant and non-pregnant women obtained by swabs (Luo et al., 2000).

Our findings on the proportions of leukocytes that were macrophages and B cells are similar to that previously reported in the sub-epithelial and stromal cervical regions (Bokstrom et al., 1997; Sakamoto et al., 2005). This is in agreement with an other immunohistochemical study of the human non-pregnant cervix reported that B-lymphocytes are few in numbers and are found only in lymphoid aggregates (Johansson et al., 1999). Tissue studies have shown similar findings, with B lymphocytes predominantly residing within the lamina propria of the endocervix and ectocervix rather than at the mucosal surface. Givan et al., (1997) stained cervical and vaginal sections for immunoglobulin-producing cells, and found that immunoglobulin-positive cells were dispersed in the lamina propria and not in the epithelial layer.

Studies on T-lymphocyte numbers in the cervix have shown conflicting results. Our observation of low numbers of T cells among the leukocytes is similar to that reported in the non-pregnant cervix and this finding distinguishes the cervical epithelial leukocyte populations from peripheral blood where there are significant numbers of T cells (Prakash M, Kapembwa M et al., 2001). The same research group has also reported that the CD4+/ CD8+ ratio in the cervical epithelium, in non pregnant women, was significantly higher than blood (Prakash M, Kapembwa M et al., 2001a). This is agreement with other studies (Levine et al., 1998; Olaitan et al., 1996). Other investigators have demonstrated the opposite result; Cohen et al.,

(1999) and Kaul et al., (2000) reported CD8+ cells to be the major T lymphocyte subset in cervix.

In our high risk pregnant women T-lymphocytes cells were scanty in the cervical mucus. Our observation is in contrast to previous publications regarding cervical leukocytes in pregnancy. Bokstrom et al., (1997) and Sakamoto et al.,(2005) described that CD3+ T-lymphocytes were the predominant lymphocyte population in the cervix, with numbers of both CD4+ and CD8+ subsets being consistently higher in the subepithelial area compared with the deep stroma. Hence, our finding of a lack of T cells in the cervical epithelium suggests that T cells are not secreted into the cervical mucus in pregnancy.

In conclusion, we have described the cervical epithelial leukocyte population in women with a past history of idiopathic preterm labour. Using the cytobrush technique, we obtained cervical mucus sample and we studied the leukocytes. The population of leukocytes compared to epithelium cells, varied significantly between patients and CD16+ granulocyte was the most common sub-population.

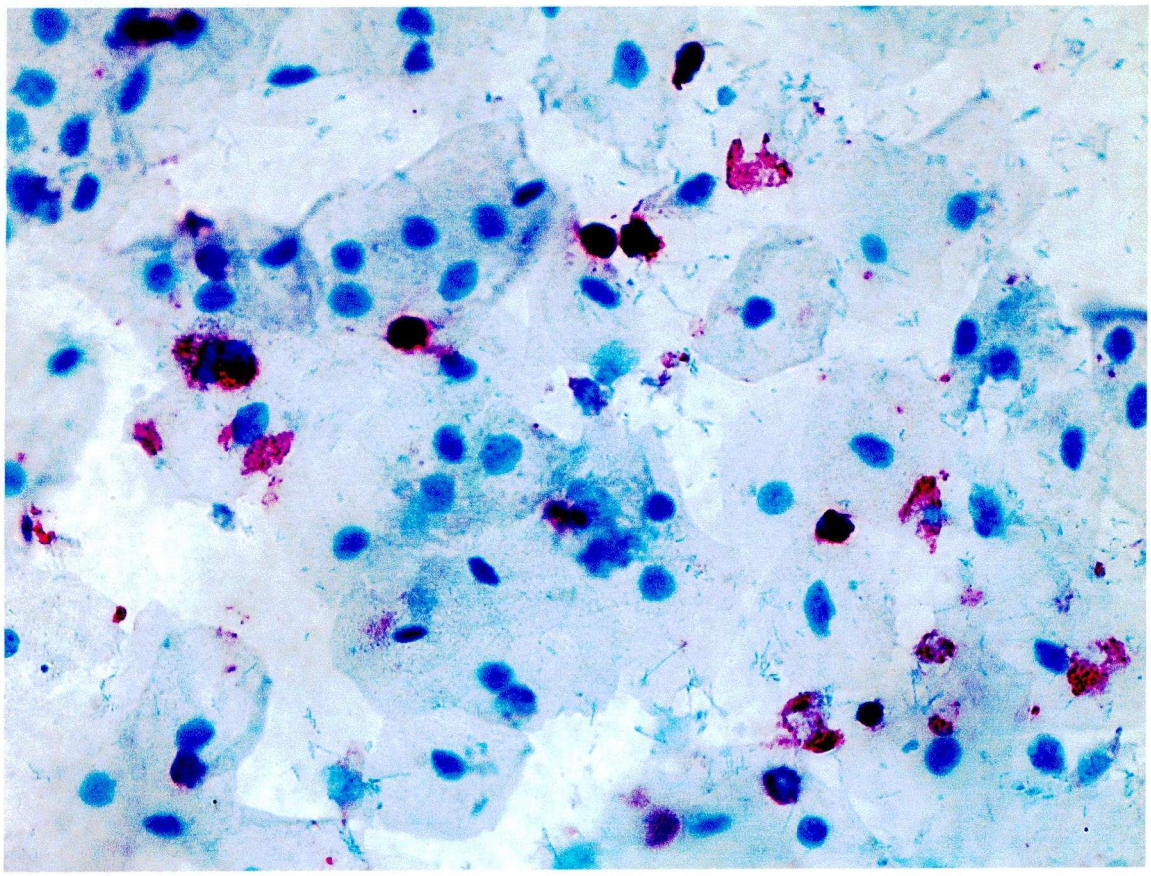


Figure 13 : Sample stained with CD16

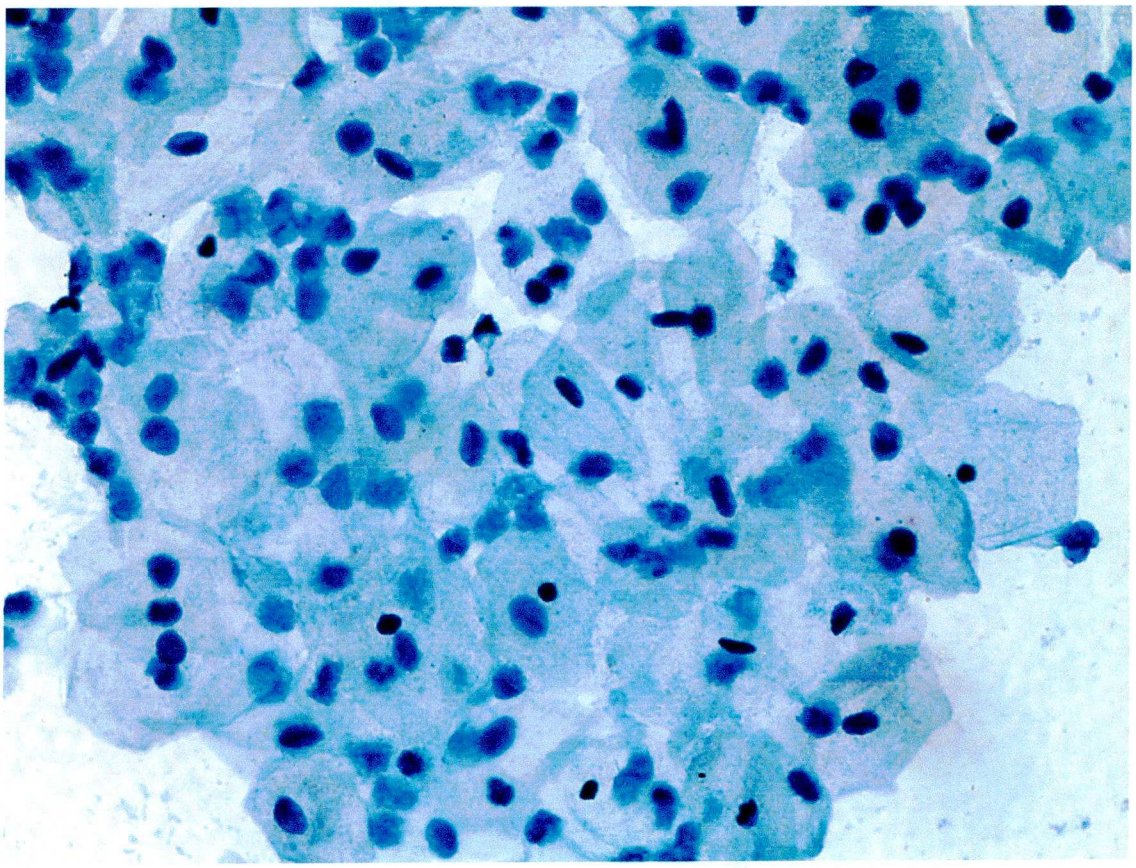


Figure 14 : Sample stained with IgG

Chapter 3

Cervical Leukocytes alteration during pregnancy.

3.1. Introduction

3.2. Methods

3.3. Data and statistical analysis

3.4. Results

3.4. Discussion

Chapter 3

Cervical Leukocytes alteration during pregnancy.

3.1. Introduction

There is now compelling evidence of a link between the cells of the reproductive tract and those of the immune system (Robertsson et al.1992). Dramatic changes have been found in the distribution of leukocytes in the ovarian tissue at ovulation and luteolysis (Brannstrom and Norman, 1993) as well as in the uterus during implantation and placentation (Pollard, 1991). These immune cells are thought to be important both for tissue remodelling and for immunosuppressive action at the feto-matemal interface.

Little is known about the possible role of leukocytes in the physiological changes in the human cervix during pregnancy and labour. In situ immunohistochemical studies have demonstrated CD4 and CD8 positive T cells, CD56 positive CD 16 negative granulated lymphocytes, and Langerhan's cells in normal ectocervical squamous epithelium (McKenzie et al., 1991). The underlying cervical stroma contains CD4 and CD8 positive T cells in varying proportions, together with classic CD56 positive, CD16 positive NK cells (McKenzie et al., 1991) and occasional macrophages (Nuovo et al., 1993). A recent flow cytometric study of lymphocytes isolated by enzyme dispersal from normal human cervix noted a predominance of B lymphocytes, while T cells and NK cells were detected at a significantly lower percentage than in peripheral blood. The majority of immunoglobulin-secreting cells produced IgG followed by IgA, with few IgM producing cells (Crowley-Nowick et al., 1995). Both the secretory (IgA mediated) and cellular immune systems are active within the cervical epithelia and stroma. Local immunity is suspected to play an important role and alterations in cervical leukocyte populations have been reported in association with HPV infection and CIN. (Blaustein's Pathology of the Female Genital Tract, 1994).

The aim of this study was to examine how the cervical epithelial leukocyte population changes during pregnancy in women with history of PTL.

3.2. Methods

Thirty five of the 81 women from our study population had their cervical leukocyte populations sampled twice during their pregnancy: at 12–16 weeks of gestation and 8 weeks later.

The group of these thirty-five women had a median age of 31.4 [range 48–19] years. Thirty two of the women were Caucasian, two Asian and one Afro-Caribbean. The median BMI was 24.8 [range 33.69–18.5]. Five of these women had a previous ectopic pregnancy and one had 2 previous ectopic pregnancies. Six women had a previous miscarriage and six had two previous miscarriages. Eight women had a previous second trimester miscarriage and three had two previous second trimester miscarriages. Seven women had a previous term delivery and three had two previous term deliveries (Table 3.1).

Table 3.1. Summary of patient characteristics (n=35)

Age (range)	31.4 (48-19)
BMI (range)	24.8(33.7-18.5)
Number underweight, BMI < 20	0
Number obese, BMI > 35	2
Ethnicity	
Caucasian	32
Afro-Caribbean	1
Asian	2
Number of women with previous ectopic pregnancies	
one previous ectopic pregnancy	5
two previous ectopic pregnancies	1
Number of women with previous early miscarriages	
one previous early miscarriage	6
two previous early miscarriages	6
Number of women with previous second trimester miscarriages	

one previous second trimester miscarriage	8
two previous second trimester miscarriages	3
Number of women with previous term deliveries	
one previous term delivery	7
two previous term deliveries	3

We obtained cervical mucus sample from these 35 women, during the first and second trimester of their pregnancy, using a fine cervical cytobrush (Cervex-brush, Rovers Medical Devices, Oss, Netherlands), as described previously in chapter 2.

Then, we used the same procedure and immunohistochemical method which has been described in chapter 2, to evaluate the cervical leukocytes sub-populations.

3.3. Data and statistical analysis

Statistical analysis was performed with Stats Direct software (Cheshire, UK). We analysed the mean of CD45+ cells (total leukocytes) as a fraction of total dispersed cells and the samples have been divided in two groups: the first group composed from the samples which have been obtained during the first trimester; the second group composed from the samples which have been obtained during the second trimester of the pregnancy.

Differences in the number of leukocyte sub-type per total cells between two groups were analysed using Mann–Whitney's U-test. Significant differences were assumed to be achieved with $P<0.05$.

3.4. Results

Leukocytes were found in every sample tested, using an antibody against antigen CD 45 present on all leukocytes. No staining was observed in negative controls with mouse IgG.

In the samples from the first trimester the mean of total leukocytes as percentage of total dispersed cells was 11.6%. After the incubation with the appropriate primary antibody, we could evaluate the subpopulations of the leukocytes in every sample. CD16+ granulocyte were the predominant subpopulation of leukocytes [5.1 % of all the cells]. The numbers of CD20+ was 0.35 % of all the cells. CD3+, CD14+, CD56+ and CD69+ were 0.1%, 0.2%, 0.2% and 0.1% of all the cells respectively.

In the samples from the second trimester the mean of total leukocytes as percentage of total dispersed cells was 7.4%. CD16+ granulocyte were again the predominant subpopulation of leukocytes [3.1% of all the cells]. The numbers of CD20+ was 0.5% of all the cells. CD3+, CD14+,CD56+ and CD69+ were 0.1%, 0.1%, 0.1% and 0.1% of all the cells respectively. There was no significant difference in leukocyte populations between the first and second samples. Comparative results for early and late pregnancy are shown in Table 3.2 and in chart 2.

Table 3.2 First and second trimester leukocyte populations

	Percentage leukocytes/total cells median		
	Median (inter quartile range) n=35		
	First sample	Second sample	P-Value
	12–16 weeks of gestation	20–24 weeks of gestation	
Leukocyte antigen			
CD45	11.6 (4.85–21.9)	7.4 (2.8–34.1)	0.93
CD3	0.1 (0–0.5)	0.1 (0–0.5)	0.84
CD4	0 (0–0.3)	0 (0–0.3)	0.86
CD8	0 (0–0.3)	0 (0–0.2)	0.79
CD14	0.2 (0–1.2)	0.1 (0.1–0.3)	0.05
CD16	5.1 (1.2–9)	3.1 (0.4–14.8	0.57
CD20	0.35 (0.1–0.3)	0.50 (0.1–0.7)	0.91
CD56	0.2 (0–1)	0.1 (0–0.7)	0.79
CD69	0.1 (0–0.3)	0.1 (0–0.3)	0.54

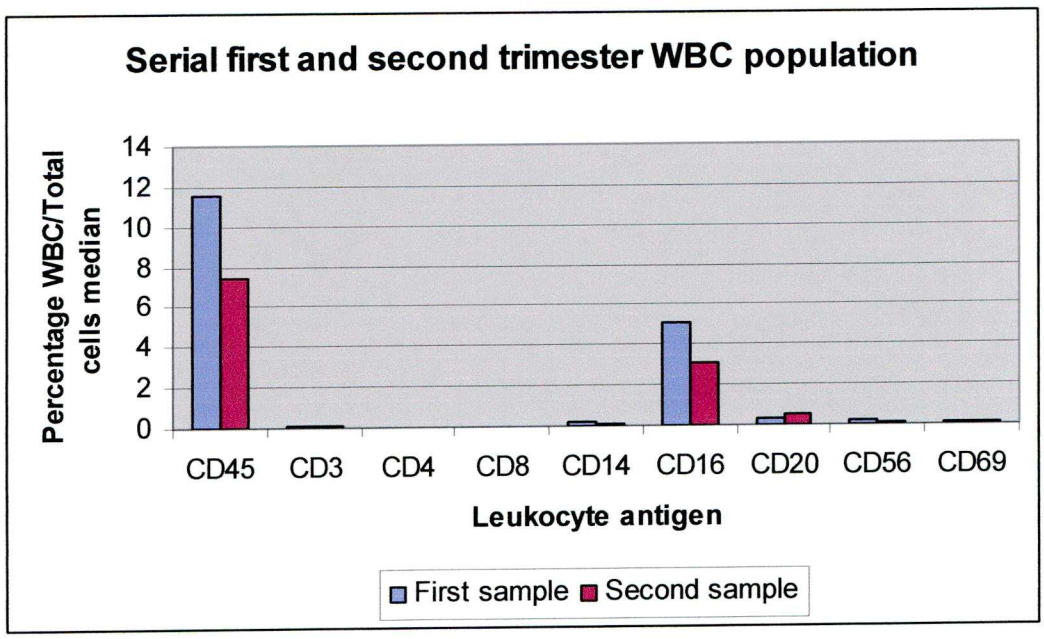


Chart 2: First and second trimester WBC population

3.4.Discussion

Our study demonstrates how the cervical epithelial leukocyte population changes in pregnancy. Our study group was thirty five women with history of previous idiopathic preterm labour. We sampled the cervical mucus of these high risk group women in the first and second trimester of their pregnancy. Using immunohistochemistry we evaluated the cervical leukocyte populations. The results indicate that the CD16+ granulocyte were the predominant subpopulation of leukocytes both in early and late pregnancy. In addition, as the gestational age increases, there is no significant difference in leukocyte populations. We are not aware of any other study on evaluation of the leukocytes population in pregnant women with history of previous PTL.

Many studies have tried to describe the alteration of the cervical leukocytes in early and term pregnancy.

In animal studies, the involvement of the cervical leukocytes in pregnancy and parturition is conflicting. Bassen 1962 and Luque et al. 1989 reported that in hedgehogs and rats eosinophils have been demonstrated in the cervical tissue in late pregnancy. Others have reported few eosinophils in rats during pregnancy and no increase during labour (Duchesne et al., 1992). Osmers et al. (1992) have shown that most collagenase involved in cervical ripening during parturition is derived from polymorphonuclear leukocytes and not from fibroblasts present in the cervical stroma. Histologically the collagen fibres seem to disappear primarily in the areas close to the eosinophils (Tchernitchin et al. 1989). Eosinophils have been found to contain a metalloproteinase that can degrade type I and III collagens (Hibbs et al. 1982), which are the two main types of collagen in the human cervix.

Using conventional histological techniques, Junqueira et al. (1980) showed a high density of polymorphonuclear neutrophil granulocytes (neutrophils) in the human cervix during the intrapartum period, and that this invasion of neutrophils was associated with breakdown of collagen fibres. Using electron

microscopy, active degranulation of these leukocytes was also observed (Junqueira et al., 1980). Ludmir et al., (2000) have also published that neutrophils are responsible for most of the connective tissue changes that take place during cervical ripening, but macrophages are also involved, although their specific role is still unclear. In agreement with this report, Bokstrom et al., (1997) supported that there is an infiltration of white blood cells (both neutrophils and macrophages) into the cervix in women at term. Specifically, they reported a 10-fold increase of the number of macrophages in cervical tissue from early to late pregnancy, but no further changes during labour.

Moreover, chemokines have been proposed to be involved in cervical ripening through their chemoattractant and activating effects on neutrophils and monocytes (Kayisli et al., 2002). Although functional redundancy exists with other chemokines in vitro, MCP-1 alone is responsible for mononuclear cell infiltration in several inflammatory animal models in vivo (Lu et al., 1998). It is thus possible that MCP-1 is also involved in the process of cervical ripening, because it is a key chemokine in the activation and recruitment of monocytes and macrophages.

The disagreement of some of our findings with the results of others studies, could reflect the difference in the mechanisms involved in cervical ripening in pregnant women with history of PPTL, from those in pregnant women who don't have such antenatal history. In addition, we have examined the leukocyte population in the first and second trimester of pregnancy and not during the intrapartum period, something that most studies have done.

However, our findings agree with previous studies that found an increase in macrophages and the chemokine IL-8 after labour has started (Osman et al., 2003; Sakamoto et al., 2005). Osmann et al., (2003) described an influx in the number of leukocytes in the cervix during labour that is caused primarily by increased numbers of neutrophils and macrophages but not T (CD3 + cells) or B-cells (CD20 + cells).

Sakamoto et al., (2005) found that macrophages and granulocytes may be involved in the process of cervical dilatation, but macrophage infiltration into the ripening cervix before labour suggests their role in the ripening process. Reduced numbers of CD3+ CD8+ T-lymphocytes in late pregnancy and after vaginal delivery suggests that local immunity is down-regulated in the late pregnancy period. They also suggest that B-lymphocytes and plasma cells are not involved in the process of cervical ripening or dilatation, but further studies are needed to clarify the role of NK cells in the process (Sakamoto et al., 2005).Our results are also supported by the study of Knudsen et al.,(1997) where no eosinophils, or very few were seen in the biopsies from non-pregnant, early pregnant women or planned caesarean section. The group assumed that eosinophils participate in cervical ripening at term in women, and seem to arise mainly after labour. Moreover, our findings are consistent with those of Timmons and Mahendroo (2006) who used immunohistochemistry to examine murine cervices both during pregnancy and during and after parturition. Leukocytosis was observed only after labour had commenced and did not precede labour (Timmons and Mahendroo, 2006).

Summarizing, the purpose of this study was to investigate how the cervical epithelial leukocyte population changes in pregnancy. Sampling the cervical mucus of our high risk pregnant women twice at 8-week intervals, we found that there was no significant difference in leukocyte populations between the first and second samples.

Chapter 4

Transvaginal ultrasound results and leukocyte population.

4.1. Introduction

4.2. Methods

4.3. Data and statistical analysis

4.5. Results

4.6. Discussion

Chapter 4

Transvaginal ultrasound results and leukocyte population.

4.1. Introduction

Changes in cervical effacement and dilatation occur well before preterm birth and must precede all spontaneous deliveries (Wood et al., 1965; Anderson et al., 1969; Papiernik et al.,1986; Blondel et al., 1988). Besides, there is also considerable evidence to show that in the absence of uterine contractions transvaginal sonographic measurement of cervical length is an effective way of identifying pregnancies at high-risk of preterm labour. Many researchers have indicated a high sensitivity and a low false positive rate for the length measurement in women at risk of preterm birth and they have indicated a significant relationship between the occurrence of preterm labour and ultrasonographical parameters (Gomez et al., 1994; Berghella et al., 1997). Hoesli et al., (1999) showed that transvaginal ultrasonography is an objective, reproducible and reliable method to assess cervical length and one of the best diagnostic markers correlating with preterm delivery, as there is an inverse correlation between cervical length and the frequency of preterm delivery in high risk patients.

Thus, in the absence of uterine contractions, there are specific ultrasonographic measurements of the cervix, best obtained via transvaginal sonography, that closely correlate with the risk of PTD. These include:

- dilatation of the internal os;
- cervical length; and
- sacculation or prolapse of the membranes into the cervix, either spontaneously or induced by transfundal pressure; this is also known as 'funnelling' (Figure 15).

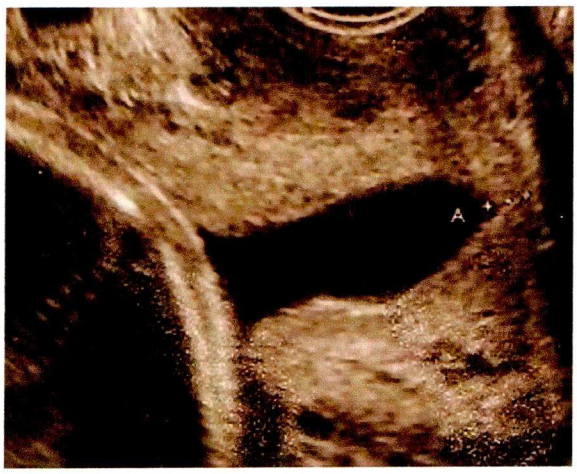


Figure 15: Cervical shortening and funnelling (A:cervical length)[www.ivfinfertility.com/.../miscarriage3.php]

It is consistent finding in many studies that the predictive power of a short cervical length or of dilatation of the internal cervical os, as assessed by transvaginal ultrasonography, has a sensitivity that ranges between 73% and 100% and a specificity that ranges between 44% and 94% in patients admitted for preterm labor (Berghella et al., 1997; Guzman et al., 2001; Heath et al., 1998; Iams et al., 1996). Honest et al., (2003), in their systematic review showed that for asymptomatic women at or below 20 weeks, a cervical length of 25 mm or less had a test positive likelihood ratio of 6.29 (95% CI 3.29–12.02) and negative test likelihood ratio of 0.79 (95% CI 0.65–0.95) for predicting spontaneous PTD before 34 weeks.

In addition, the relationship between cervical length during pregnancy and increased risk of perinatal infection has been the subject of study in the prediction of prematurity. A short cervix has been considered to be an independent risk factor for the subsequent development of clinical chorioamnionitis and subsequent preterm labour (Iams et al., 1997). Romero R, Gonzalez Ret al, (1992) reported that microbial invasion of the amniotic cavity was present in 55.1% (17/33) of patients presenting between 14 to 24 weeks of gestation with a cervical dilatation >2 cm, intact membranes, and without active labour (Romero et al., 1992).

Hence, the sonographic cervical changes are anatomic manifestations of altered cervical physiologic factors and represent a final common pathway for

multiple pathologic processes. The aim of our study was therefore, to investigate the hypothesis that a cervical leukocytosis was a prelude to cervical shortening and funnelling.

4.2. Methods

In this prospective study, we examined the leukocyte sub-populations of our high risk study group (n=81) which we have described in chapter 2, in correlation with the cervical shortening and funnelling. The demographic characteristics and obstetrics history of the study group are shown in table 2.1. The sampling method and the technique of the leukocyte evaluation have already been described in chapter 2. As some woman delivered prior to the second sampling, the first sample (12-16 weeks of gestation) was determined to be the optimal time for the prospective study.

As per clinic protocol, all women had serial cervical length measurement using transvaginal ultrasonography at least at 12, 16, 20, 24 and 28 weeks of gestation. The ultrasound examination was performed in a standardised manner with a stress test of the cervix (Shennan and Jones, 2004). After the patient's bladder was emptied, the probe was gently placed in the anterior fornix of the vagina to obtain a sagittal view of the complete cervix, including external and internal os and the cervical canal. Then the probe was slightly withdrawn and positioned without pressure on the cervix to obtain a clear picture. Care was taken to ensure that in the sagittal view the distance from the surface of both anterior and posterior lip to the cervical canal was equal. For a stress test, mild abdominal pressure was applied for 30 seconds whilst simultaneously scanning the cervix so, all patients had cervical measurements obtained with and without transfundal pressure as described by Guzman et al., 1994. Two parameters were determined: 1) the cervical length which was measured as the distance between the internal and external os, identified by the sonolucency of the cervical canal and 2) whether funnelling occurred, defined as a 'v'- or 'u'- shaped indentation of the internal

os by the amniotic membranes. (Fig. 15). Because pressure against the cervix generated by insertion of the transducer can influence the length of the endocervical canal, it was measured 3 times at each session. When different values were obtained, the minimum value was used, consistent with previously described methods (Iams et al., 1996; Iams et al., 1994; Guzman et al., 1994). All ultrasound measurements were performed by a single investigator.

4.3. Data and statistical analysis

Statistical analysis was performed with Stats Direct software (Cheshire, UK). We analysed the mean of CD45+ cells [total leukocytes] and leukocyte sub-populations as a fraction of total dispersed cells and the samples were divided in two groups: the first group composed from women who developed cervical changes: a positive stress test (the present of cervical funnelling upon application of fundal pressure), or cervical shortening <25 mm; the second group composed from women who did not develop either of these cervical changes.

Differences in the number of leukocytes sub-type per total cells between the two groups were analysed using Mann–Whitney's U-test. A p-value <0.05 was considered significant.

4.5. Results

Serial transvaginal ultrasonography until 28 weeks of gestation identified that fifty-eight of our eighty-one women didn't develop any cervical change. 23 women had a positive stress test (cervical funnelling upon application of fundal pressure). 9 of them also developed cervical shortening <25 mm.

The group of these 23 women had a median age of 28.4 [range 40-21] years. 17 of them were Caucasian, 5 Afro-Caribbean and 1 Asian. The average BMI was 26.7 [range 39-21]. Eight of these women had a previous ectopic pregnancy and three had 2 previous ectopic pregnancies. Six women had a previous miscarriage and four had two previous miscarriages. Three women had a previous term delivery and two had two previous term deliveries (Table 4.1).

Table 4.1 Summary of patient characteristics (n=81)

	Cervical change (n=23)	No Cervical change (n=58)
Age (mean years)	28.4 (range 40-21)	28.72 (48-19)
BMI (mean)	26.7 (39-21)	24.46 (45-20)
Ethnicity		
Caucasian	17	49
Afro-Caribbean	5	5
Asian	1	4
Number of women with previous ectopic pregnancies		
one previous ectopic pregnancy	8	7
two previous ectopic pregnancies	3	2
Number of women with previous early miscarriages		
one previous early miscarriage	6	15
two previous early miscarriages	4	5

Number of women with previous term deliveries		
one previous term delivery	3	9
two previous term deliveries	2	4

The group of the 58 women who did not develop any cervical change had an average age of 28.72 [range 48-19] years. Forty-nine of them were Caucasian, five Afro-Caribbean and four Asian. The average BMI was 24.46 [range 45-20]. Seven of these women had a previous ectopic pregnancy and two had two previous ectopic pregnancies. Fifteen women had a previous miscarriage and five had two previous miscarriages. Nine women had a previous term delivery and four had two previous term deliveries (Table 4.1).

In the cervical mucus samples obtained from the women who developed cervical changes the mean of total leukocytes as percentage of total dispersed cells was 10.65%. CD16+ granulocyte was the predominant sub-population of leukocytes [3.48 % of all the cells]. The numbers of CD20+ was 0.12 % of all the cells. CD3+, CD14+, CD56+ and CD69+ were 0%, 0%, 0.35% and 0.1% of all the cells respectively.

In the samples from women who didn't develop any cervical change the mean of total leukocytes as percentage of total dispersed cells was 19.4%. CD16+ granulocyte was again the predominant sub-population of leukocytes [9.73% of all the cells]. The numbers of CD20+ was 0.45% of all the cells. CD3+, CD14+, CD56+ and CD69+ were 0%, 1.8%, 0.2% and 0.1% of all the cells respectively.

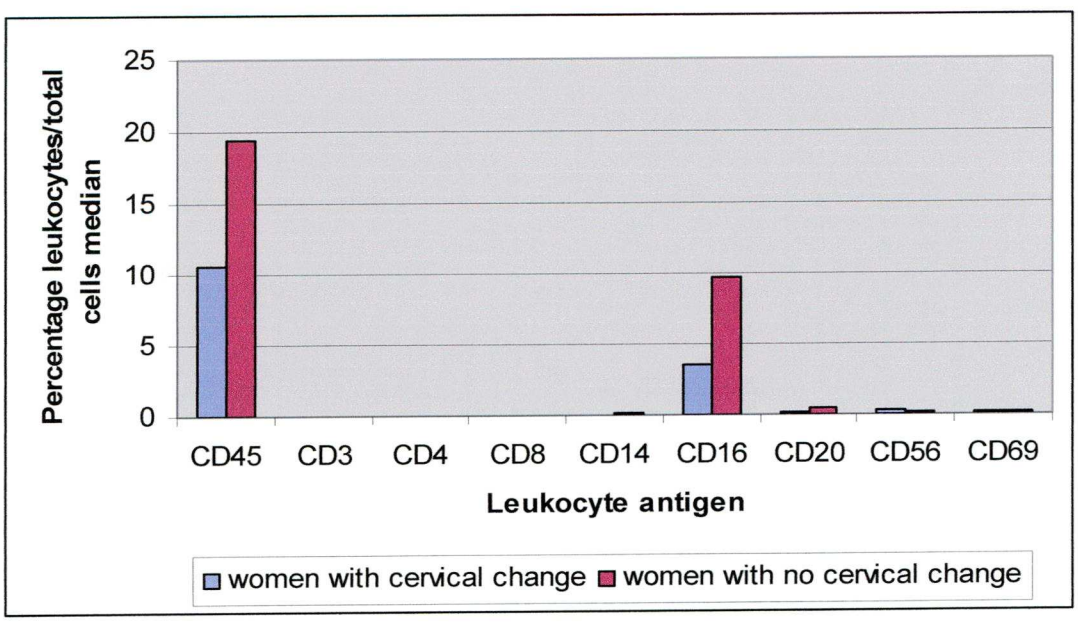
After analyzing the results, we found that there was no significant difference in leukocyte populations between the first and second sample groups. Comparative results between the two groups are shown in (Table 4.2 and in chart 3).

Table 4.2 Leukocyte populations between the two groups of the study

Percentage leukocytes/total cells median (interquartile range)

Leukocyte antigen	women with cervical change (n=23)	women with no cervical change (n=58)	P-Value
CD45	10.65 (2.95-29.85)	19.4 (3.12-30.27)	0.77
CD3	0 (0-0.32)	0 (0-0.21)	0.71
CD4	0 (0-0.21)	0 (0-0.23)	0.85
CD8	0 (0-0.24)	0 (0-0.3)	0.78
CD14	0 (0-1.38)	0.18 (0-0.77)	0.47
CD16	3.48 (0.41-11.85)	9.73 (1.41-18.42)	0.2
CD20	0.12 (0-1.4)	0.45 (0.1-3.5)	0.12
CD56	0.35 (0-0.56)	0.2 (0-0.7)	0.25
CD69	0.1(0-0.3)	0.1(0-0.3)	0.89

Chart 3: Leukocyte populations between the two groups of the study



4.6. Discussion

A wealth of evidence supports the connection of cervical effacement and dilatation to risk of labour. Many studies of cervical change during pregnancy have reported that preterm effacement and dilatation is a normal process, more pronounced in multipara than nulliparous women (Parikh et al., 1961; Floyd et al., 1961; Schaffner et al., 1966). Andersen et al., (1990) were the first to report that ultrasound cervical length measurements are inversely related to risk of preterm birth. Tongsong et al., (1995) confirmed these findings using endovaginal ultrasound at 28 to 30 weeks of gestation.

We today know that ultrasound examination of the cervix in patients in preterm labour can identify patients likely to progress to preterm delivery as an inverse relationship between cervical length and preterm delivery has been well documented (Iams et al., 1996; Welsh et al., 2002; Owen et al., 2001; Hibbard et al., 2000; Leitich et al., 1999). Additional studies show that cervical shortening or dilatation predicts an increased risk of preterm delivery and helps us to view the preterm labour as a continuum both temporally throughout gestation and causally as a result of numerous interacting factors and circumstances. Premature cervical shortening is associated with a 1.5- to 3-fold increased risk of preterm delivery (Leveno et al., 1986; Stubbs et al., 1986; Papiernik et al., 1986; Mortensen et al., 1987; Neilson et al., 1988; Catalano et al., 1989). Bartolucci et al., (1984) evaluated 48 women presenting with premature labour. They described four specific sonographic findings associated with the risk of preterm delivery: shortening of the cervix to less than 3 cm (52 percent risk of preterm delivery), dilatation of the endocervical canal (41 percent risk), bulging fetal membranes (funneling, 42 percent risk); and thinning of lower uterine segment (40 percent). Using endovaginal ultrasound, Murakawa et al. (1993) studied 32 women with singleton pregnancies and in preterm labor. Cervical length was measured at the time of admission. The risk of preterm delivery increased with decreasing cervical length. All women with cervical lengths of less than 20 mm

delivered prematurely. Iams et al, (1996) studied 60 women, including 12 twin pregnancies, who were being treated for preterm labour. There were no births before 36 weeks in women with cervical lengths of more than 30mm measured by ultrasound. These results are comparable to a systematic review published in 2003 which showed a likelihood ratio (LR) of 6.29 (95% CI 3.29–12.02) for preterm birth before 34 weeks in women with a cervical length of < 25 mm at 20 weeks or earlier, thus converting the pre-test probability of preterm delivery (before 34 weeks) of 4.1% to a post test probability of 15.8%. Funnelling of the cervix seems to increase the risk of preterm delivery, with the risk increasing progressively with increasing degree of funnelling (Honest et al., 2003).

Cervical funnelling also develops in patients with preterm labour (Gomez et al., 1994). In such women cervical funnelling appears to be more transient. Dynamic cervical change involving 50 percent or more of the cervical canal is associated with incompetent cervix or preterm delivery. These observations support a hypothesis of two overlapping phenomena: uterine activity and cervical function. The classical patient with incompetent cervix is at the far end of the spectrum of cervical performance such that her internal os dilates with minimal uterine activity. In this case it is reasonable that funnelling or dynamic change would develop over several days or even weeks due to the low level of normal uterine activity in early gestation. Once the stromal changes of the cervical ripening occur, dilatation of the internal os can occur rapidly.

Moreover, cervical incompetence is a clinical diagnosis traditionally defined as painless cervical dilatation in the second trimester with prolapse and ballooning of the membranes into the vagina, which is usually followed by rupture of membranes and expulsion of an immature fetus. This sequence of events is repeated frequently in subsequent pregnancies and its characteristic is that the cervical dilatation and effacement have occurred in the absence of a significant increase in uterine contractility (Abortion, In : Williams obstetrics; 2001).

Diagnosis has been based on history and clinical examination. Other diagnostic tests have included pull-through techniques with catheter balloons or cervical dilators (Ansari et al., 1987) and more recently, ultrasound scanning. Mahran (1980) reported that an internal os diameter of more than 1.5 cm in the first or second trimester was associated with incompetent cervix. Vaalamo and Kivikoski (1983) and Bernstine et al. (1981) emphasized the ultrasound observation of membranes herniating or bulging into a partly dilated cervix as the criterion for diagnosis. Vamla et al., (1986) observed a cervical canal dilatation of more than 8mm in patients who required cervical cerclage. Iams et al. (1995) have suggested that cervical incompetence is not an "all or none" phenomenon but rather a continuum. They postulate a multifactorial model of spontaneous preterm birth that suggests that one or more pathophysiologic processes may act on an otherwise competent cervix to convert it into a distendable conduit through which preterm birth can take place. These ultrasonographic signs overlap with diagnostic criteria for cervical incompetence, and can be detected in early pregnancy as well as midtrimester. This implies that cervical competence may exist as a continuum rather than a dichotomous entity, as previously believed.

In summary, the recommendations for cervical changes as a screening test are based on the fact that sonographical assessment is a widely accepted and well standardised method. The concept of a continuum of cervical competence is useful in considering the contribution of the cervix to the genesis of prematurity.

Our objective was to examine prospectively the relation between cervical dilatation and length and the cervical subtypes of leukocytes in a high risk for preterm labour population. Our study group was eighty one women with history of previous idiopathic preterm labour.
Using the cytobrush technique, we obtained their cervical mucus samples and after the incubation with the appropriate primary antibody, we could evaluate the leukocyte sub-population in every sample. We performed serial transvaginal ultrasonography until 28 weeks of gestation measuring the cervical length and funnelling. The examination was well accepted by

pregnant women as demonstrated in a prospective study (Heath et al., 1998). A single ultrasonographic operator performed all cervical length measurements to eliminate the possibility for inter- observer variability in measurement technique.

We identified 23 women who had a positive stress test (cervical funnelling upon application of fundal pressure) and 9 who developed cervical shortening <25 mm. The number of patients and mean number of cervical leukocytes for each group were recorded and are summarized in Table 4.2.

Our immunohistochemistry results indicate that the CD16+ granulocyte were the predominant subpopulation of leukocytes both in women who subsequently developed either of these cervical changes and in those who did not. Additionally, there were no differences in leukocyte populations in women who subsequently developed either of these cervical changes compared to those who did not.

In summary, we have found that cervical leukocytosis was not a prologue to cervical shortening and funnelling.

CHAPTER 5

Cervical leukocyte population and risk of recurrent preterm delivery

5.1 Introduction

5.2 Methods

5.3 Data and statistical analysis

5.4 Results

5.5. Discussion

CHAPTER 5

Cervical leukocyte population and risk of recurrent preterm delivery

5.1 Introduction

Clinical and experimental evidence indicates that preterm labour results from a pathophysiologic cascade described by Lockwood and Kuczynski (1999). The common final biochemical pathway that initiates parturition involves myometrial activation and stimulation and cervical changes. Cervical ripening occurs prior to dilatation, is a prerequisite for vaginal delivery and is associated with collagen remodelling and altered proteoglycan and water content (Junqueira et al., 1980; Osmers et al., 1993, 1995).Absence of normal ripening at term is associated with prolonged labour and post-term pregnancy, whereas premature ripening occurs as part of the preterm delivery syndrome or a second trimester abortion (Olah and Gee, 1992).

Cervical ripening has already been described as an inflammatory process (Sennstrom et al., 2000). A convergence of evidence suggests that inflammatory processes that involve prostaglandins (Calder et al, 1991), nitric oxide (Buhimschi et al, 1995) and cytokines (Colditz et al, 1990), may enhance uterine contractility, promote ripening of the cervix and thus constitute an integrative hypothesis for the initiation of labour (Yellon et al, 2003). Moran et al. (2003) showed that the cervical dilatation is accompanied by a granulocyte, macrophage and NK cell infiltrate. Recent series of studies have shown that preterm parturition is associated with an increase in interleukin IL-6 and IL-8 and monocyte chemotactic protein-1 concentrations, and a decrease in prostaglandin dehydrogenase, even in the absence of infection (Tornblom SA, Klimaviciute A et al., 2005).

Although genital tract infections increase the risk of PTL (Goldenberg et al, 2003), (McDonald et al, 1991), some studies say that the treatment of the infection does not decrease the risk of preterm delivery (Carey et al, 2003).

Our initial hypothesis was that excessive leukocyte response to sub-clinical alteration of the vaginal flora causes cervical ripening rather than infection itself. Furthermore, the aim of this study was to investigate the supposition that recurrent preterm labour is associated with a different cervical leukocyte population.

5.2 Methods

In this prospective study, we examined the leukocyte subpopulations of our 81 high risk pregnant women which we have described in chapter 2, in correlation with the pregnancy outcome.

The demographic characteristics and obstetrics history of the study group are shown in table 2.1.

Our group of eighty-one women had their cervical mucus sampled in the first trimester of gestation and their leukocyte populations evaluated, using the immunohistochemical method which has been described in chapter 2.

We tried to find any possible connection between cervical leucocytes and the time of the delivery.

5.3 Data and statistical analysis

Statistical analysis was performed with Stats Direct software (Cheshire, UK). We analysed the mean of the different type of leukocytes as a fraction of total dispersed cells. We divided our group in two sub-groups, the first consisted of women with recurrent spontaneous preterm labour before 35 weeks gestation (n=24) and the second of women with non-recurrent preterm labour (n=57).

Differences in the number of leukocytes sub-type per total cells between two groups were analysed using Mann–Whitney's U-test. Significant differences were assumed to be achieved with $P<0.05$.

5.4 Results

In our study group, twenty four of the eighty one women went into spontaneous preterm labour and delivered prior to 35 weeks of gestation (range 22–34 weeks gestation), but only four of these delivered prior to 28 weeks of gestation. Fifty-seven women delivered after 35 weeks of gestation.

The demographics and the patients' past obstetric history is shown in Table 5.1.

Table 5.1 Summary of patient characteristics (n=81)

	Recurrent Preterm delivery (= 24)	Delivered after 35 weeks gestation (= 57)
Age (range)	30.2 (48-25)	31(40-19)
BMI (range)	26 (21-40)	25 (21-45)
Ethnicity		
Caucasian	21	45
Afro-Caribbean	2	8
Asian	1	4
Number of patients who had a previous birth before 28 weeks'	14	34
Number of patients who had a previous neonatal death due to prematurity	10	28
Number of patients with an existing child with cerebral palsy due to prematurity	2	0

The group of women delivered prior to 35 weeks of gestation had a median age of 30.2 years. Twenty one of them were Caucasian, two Afro-Caribbean and one Asian. The average BMI was 26 (range 21-40). 14 (58%) women had a previous delivery prior to 28 weeks of gestation, 10 (41%) had suffered at least one neonatal death due to prematurity and two (8%) had children that survived premature birth but were affected by cerebral palsy.

The group of women who subsequently had a term labour had a median age of 31 years. Forty-five of them were Caucasian, eight Afro-Caribbean and four Asian. The average BMI was 25 (range 21-45). 34 (60%) women had a previous delivery prior to 28 weeks of gestation and 28 (49%) had suffered at least one neonatal death due to prematurity.

The median numbers of the major cervical leukocyte populations, in women who delivered prior to 35 weeks gestation and those who delivered after 35 weeks of gestation, are shown in Table 5.2.

Table 5.2 First trimester cervical leukocyte populations for women with recurrent and non-recurrent preterm labour.

Percentage leukocytes/total cell median (inter quartile range)

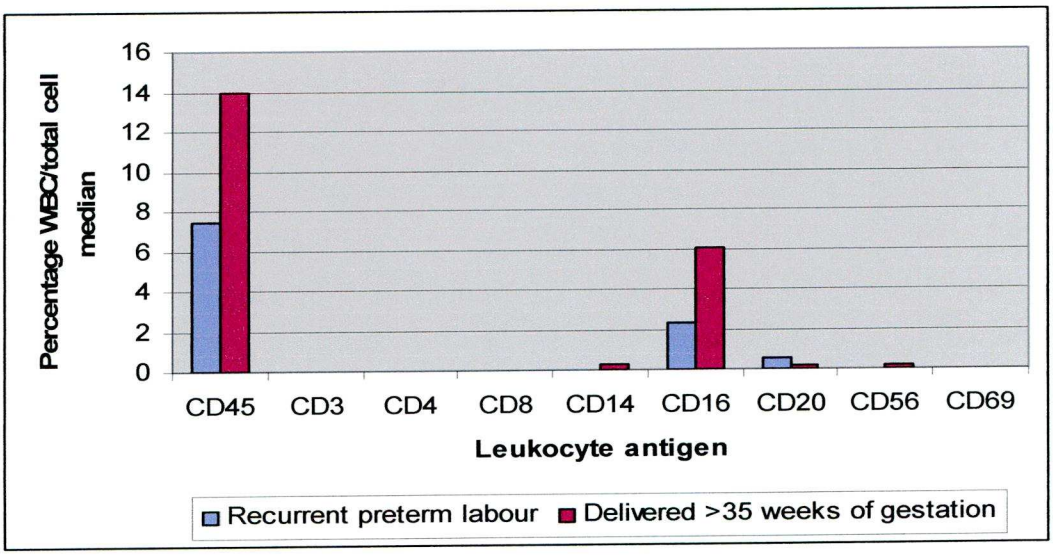
Leukocyte antigen	Recurrent preterm labour (= 24)	Delivered >35 weeks of gestation (= 57)	P-Value
CD45	7.4 (3.17–20.14)	13.92 (4.68-29.35)	0.26
CD3	0 (0-0.18)	0 (0-0.16)	0.73
CD4	0 (0-0.19)	0 (0-0.30)	0.84
CD8	0 (0-0.03)	0 (0-0.27)	0.69
CD14	0 (0-0.20)	0.26 (0.1-0.5)	0.008
CD16	2.29 (0.59-9.03)	6.08 (1.10-13.93)	0.15
CD20	0.54 (0.10- 3.54)	0.15 (0-0.15)	0.15
CD56	0 (0-0.56)	0.19 (0-1.40)	0.12
CD69	0 (0-0.11)	0 (0-0.11)	0.89

In the samples taken from those patients who subsequently had recurrence of their preterm labour and delivered prior to 35 weeks of gestation, the mean of total leukocytes as percentage of total dispersed cells was 7.4%. CD16+ granulocyte were the predominant subpopulation of leukocytes (2.29% of all the cells). The numbers of CD20+ was 0.54 % of all the cells. Moreover, there were scanty CD3+,CD4+,CD8+,CD14+ and CD56+ in this group.

In the samples taken from those patients who subsequently had a term labour the mean of total leukocytes as percentage of total dispersed cells was 13.92%. CD16+ granulocyte were again the predominant subpopulation of leukocytes (6.08% of all the cells). The numbers of CD20+ was 0.15% of all the cells. CD14+ and CD56+ were 0.26% and 0.19% of all the cells respectively.

When we compared the results, we found that there were significantly fewer CD14+ macrophages in the patients who subsequently had recurrence of their preterm labour and delivered prior to 35 weeks of gestation (p=0.008) (Table 5.2).Comparative results between the two sub-groups are shown in chart 4.

Chart 4: Cervical leukocyte populations for women with recurrent and non-recurrent preterm labour



5.5. Discussion

The present study is the first to examine the distribution of leukocyte subtypes in the human cervical mucus using immunohistochemical methods, in correlation with the pregnancy outcome, in women with history of previous preterm delivery. The cytobrush technique was successfully translated into our population of women at high risk of recurrent preterm labour, thus enabling us to examine the cervical epithelial leukocyte populations prospectively and investigate whether cervical epithelial leukocytes were related to pregnancy outcome.

Previous studies have described an increase in cervical mucus granulocytes prior to the onset of term labour. Luo et al., (2000) reported that leukocytes migrate into cervical stroma and mucus during labour reaching a density 2–3-fold higher than that found in late pregnancy.

In addition, IL-8 is elevated in cervical secretions and amniotic fluid in cases of preterm labour (Gonzalez et al., 2005; Holst et al., 2005; Wennerholm et al., 1998). Because interleukin-8 is a potent neutrophil chemoattractant (Kuipjers et al., 1992), cervical neutrophil counts could reflect local interleukin-8 concentrations.

Moreover, in cervicovaginal fluid, significant associations between elevated cytokine levels and preterm birth have been described previously for: IL-1 (Gonzalez et al.,2005; Coleman et al., 2001; Kalinka et al., 2005), IL-6 (Goepfert et al., 2001; Lange et al., 2003; Gonzalez et al., 2005; Coleman et al., 2001; Kalinka et al., 2005), IL-8 (Coleman et al., 2001; Dowd et al., 2001; Gonzalez et al., 2005; Kalinka et al., 2005) and IL-18 (Jacobsson et al., 2003). Immunohistochemical analyses of cervical biopsies have shown that IL-1 is produced predominantly by leukocytes, IL-6 by leukocytes, glandular epithelial cells and surface epithelial cells, and IL-8 is produced primarily by leukocytes, glandular epithelial cells, surface epithelial cells and stromal cells (Young et

al., 2002). Following, these pro-inflammatory cytokines can induce the ripening of the cervix in a number of ways (Watari et al., 1999 ;Sato et al., 2001 ; Kelly 2002).

Many authors have supported the theory that ascending genital tract infection stimulates a host immune response that, in turn, could initiate labour partly due to a bystander effect (Kenyon et al., 2001; Romero et al., 2002; Winkler, 2003). Our study was designed to produce evidence to support this theory. Our findings contrast with this hypothesis, as we can see in table 5.2. We did not find the elevated number of granulocytes that would be expected if these women went into recurrent preterm labour because of an excessive host response to genital tract flora early in pregnancy.

However, in our study the sampling occurred 1–3 months prior to labour; thus, it is possible that sampling was too early to detect an increase in cervical mucus leukocytes close to preterm labour. In agreement with this explanation, there was low rate of infection among our population of women (only 15 of 106).

Women with a poor obstetrics history, especially history of premature delivery, will seek medical attention regarding vaginal discharge in the postnatal period or prior to conceiving. We were not able to ascertain if any of our women had clinic or GP visits for common genital tract infection screening prior to pregnancy. For this reason, we lacked knowledge about specific treatments that might lead to a reduction in the vaginal bacterial load in their early pregnancy and hence to reduced numbers of macrophages in cervical epithelium.

It is also possible that women with preterm labour develop abnormal vaginal flora later in pregnancy and an inflammatory response which includes high numbers of leukocytes in the cervical epithelium.

However, a number of observations suggest an alternative explanation for our findings. Results of previous studies indicated that leukocyte influx into

the cervix follows cervical dilatation in labour and is not a feature of cervical ripening or the initiation of labour (Timmons and Mahendroo, 2006).

In addition, our observations are in keeping with the findings of many study groups, such as Sakamoto et al., (2005) and Osman et al., (2003), who demonstrated that CD45+ leukocytes, namely granulocytes and macrophages, dramatically increased after vaginal delivery, but did not increase significantly in the late pregnancy pre-labour group compared with the early pregnancy group. These studies again suggest that leukocyte infiltration of the cervix happens after the start of labour and is a result of labour. If cervical leukocytosis was a cause of labour it would need to be seen prior to the onset of labour. Moreover, these findings mirror local pro-inflammatory cytokine production as IL-8 levels also increase dramatically in both cervical stroma and epithelium after labour and vaginal delivery (Sakamoto et al., 2004).

Our study found that a specific leukocyte subtype, the macrophage (CD14+), was less common ($p < 0.01$) in the cervical epithelium in the early second trimester of pregnancy among high risk women who subsequently had a recurrent spontaneous preterm labour and delivery compared to those that delivered after 35 weeks of gestation. The team of Simhan et al., (2003) has reported that women with low concentrations of 2 or 3 cytokines (IL-1 b, IL-6, and IL-8) in the vaginal fluid in early pregnancy (at 8 to 20 weeks) are more likely to subsequently develop clinical chorioamnionitis than those without low concentrations of these 3 cytokines. The present study takes this concept further. IL-1, IL-6 and IL-8 are produced primarily by leukocytes (Young et al., 2002). Thus, limited number of macrophages may result in low concentrations of cytokines and this in tern may result in lack of protection from vaginal organisms, ascending infection, choriamnionitis and preterm delivery.

Summarizing, the analysis of the results of this prospective observational study prompt us to speculate that cervical epithelial macrophages may serve to prevent recurrent preterm labour, possibly by preventing ascending infection.

Chapter 6

Cervical leukocyte response to abnormal genital tract pathogens

6.1. Introduction

6.1.2 Normal and abnormal genital-tract flora

6.1.3 Cellular immunity in the pregnant cervix

6.2. Methods

6.3. Data and statistical analysis

6.4. Results

6.5. Discussion

Chapter 6

Cervical leukocyte response to abnormal genital tract pathogens

6.1. Introduction

Inflammation is the only cause of preterm labor for which the molecular pathophysiology has been well characterized (Romero R, Mazor M et al., 1994; Mitchell et al., 1991). Labour is a dual process involving a decrease in cervical resistance leading to progressive effacement and dilatation, accompanied by an increase in uterine contractility resulting in longer, stronger, more frequent and more synchronous contractions. Prostaglandins induce both of these processes. Prostaglandins are synthesised from arachidonic acid, which is the obligate precursor of prostaglandin synthesis. Arachidonic acid is released from glycerophospholipids in the cell membrane by specific phospholipase enzymes such as phospholipase A_2 (PLA_2). Bacterial pathogens may release phospholipases, which initiate the formation of arachidonic acid, and hence the arachidonic acid cascade of prostaglandin production (Lamont et al., 1985; Bennett et al., 1987; Lamont et al., 1990). By producing other enzymes such as proteases or mucinases, microorganisms may penetrate the cervical mucus plug. Thereafter, by producing enzymes such as collagenases or elastases, they may cause membrane disruption, which either leads to PPROM or release of PLA_2 enzymes from intracellular organelles (lysosomes) resulting in an increased release of arachidonic acid with prostaglandin synthesis (McGregor et al., 1987; Lamont RF, Fisk N., 1993; McGregor et al., 1988). Alternately, these pathogens can release endotoxins, which act on a number of cells, particularly macrophages, to cause prostaglandin or cytokine release. These proinflammatory cytokines, which include IL-1, TNF, and IL-6, in turn act on amnion cells or decidual stoma cells to increase expression of enzymes of the prostaglandin biosynthetic pathway and thereby increase eicosanoid production that induce preterm delivery (Splichal et al., 2001).

6.1.2 Normal and abnormal genital-tract flora

The vaginal flora is normally dominated by lactobacillus species, which produce lactic acid that maintains vaginal pH below 4.5. At this low pH, lactobacilli produce hydrogen peroxide (H_2O_2), which is toxic to bacteria, firstly by producing toxic hydroxyl radicals and secondly by combining with a large pool of chlorine ions in the vagina to produce chloradinium ions. If there is an increase of vaginal pH, lactobacilli lose their ability to produce H_2O_2 and the normal lactobacillus-dominated flora is replaced by a thousand-fold overgrowth of anaerobic and other organisms such as Gardnerella vaginalis, Mycoplasma hominis and Mobiluncus spp. These organisms produce keto-acids such as succinate, which blunts the chemotactic response of polymorphonuclear leukocytes and also their killing ability.

Using Gram stain of vaginal secretions, vaginal flora can be classified as grade I: normal (predominantly lactobacillus morphotypes); grade II: intermediate (reduced lactobacillus morphotypes mixed with other bacterial morphotypes); and grade III: bacterial vaginosis (few or no lactobacillus morphotypes with greatly increased numbers of G. Vaginalis and other morphotypes) (Rosenstein et al.,1996).

On the other hand, lower genital tract bacteria act locally, producing enzymes such as sialidase or mucinase, which may weaken protective cervical mucus and promote bacterial invasion of the upper genital tract (McGregor et al., 1992). Constantly, the lower the gestational age at presentation of preterm labour, the higher the frequency of positive amniotic fluid cultures (Watts et al., 1992).

6.1.3 Cellular immunity in the pregnant cervix

Amniotic fluid is generally bacteria-free in women not in labour, so that bacterial detection is likely to be significant. Even though the vagina is "polymicrobially colonized and is known to be ecological niches, with a specific transient and resident flora, the uterine cavity is usually sterile"

(Eggert-Kruse et al., 2000). One explanation for this might be the presence of local antibacterial factors in the cervical mucus. The cervical mucus is well known as a multifactorially determined filtering system, but not much is known about its antimicrobial activity (Eggert-Kruse et al., 2000). Once local infection occurs, macrophages, neutrophils, and mast cells that produce inflammatory cytokines migrate into cervical tissue. Inflammatory cytokines stimulate production of proteases that induce cervical ripening (Gibbs et al., 1992).

The neutrophil is the predominant white blood cell found in the cervix during normal parturition, as well as in the amniotic fluid, chorionic membranes, and placenta during infection-induced preterm labor (Cherouny et al., 1993). Neutrophils and macrophages are sources of collagenase, elastase, and other enzymes capable of digesting extracellular matrix proteins (Ito et al., 1997); therefore, these enzymes have an important role during cervical ripening. Thus, it would seem logical that when examining the vaginal habitat, clinicians should be interested in the microbial-host interaction. Upon recognition of the presence of invading microorganisms (or even perhaps excessive proliferation of facultative flora), the innate immune system amounts a response presumably aimed at controlling the infectious process. Macrophages, dendritic cells, and neutrophils are key to this process (Iwasaki et al., 2003; Zhao et al., 2003). Normally, these cells are quiescent and must be activated to become effective. The process of activation and deactivation of inflammatory cells is central to homeostasis (Bellingan G., 1999). A balanced and appropriate response is thought to be beneficial to clear infectious agents through the production of antimicrobial agents, phagocytosis, and clearance of microorganisms.

For this reason, the target of our study was to investigate the cervical leukocyte response to abnormal genital tract pathogens.

6.2. Methods

We studied the leukocyte subpopulations of our high risk study group (n=96) which have we described in chapter 2, in correlation with abnormal genital tract flora. The sampling method and the technique of the leukocyte evaluation have already been described in chapter 2.

As per clinic protocol, all women were screened for eight genital tract infections during their visits in our PTL clinic: Bacterial Vaginosis (BV), Gardnerella vaginalis (GV),Trichomonas Vaginalis (TV), Yeasts, Group B Streptococcus (BHEM), Chlamydia, Ureoplasma and Mycoplasma. The procedure was performed in a standardised manner. Subjects were placed in the standard position for gynaecological examination with feet in stirrups. A saline-moistened plastic speculum was inserted in the vaginal canal and the cervical os was exposed. Samples obtained using sterile cotton-tipped swabs for screening of the above infections. Chlamydia antigen, ureaplasma urealyticum and myoplasma hominis were tested for using polymerase chain reaction (PCR).

6.3. Data and statistical analysis

Statistical analysis was performed with Stats Direct software (Cheshire, UK). We analysed the mean of CD45+ cells (total leukocytes) as a fraction of total dispersed cells and the samples have been divided in two groups: the first group composed from women with abnormal genital tract flora; the second group composed from women who did not develop any genital tract infection. Differences in the number of leukocyte sub-types per total cells between the two groups were analysed using Mann–Whitney's U-test. A p-value <0.05 was considered significant.

6.4. Results

Swabs for vaginal infection identified 81 of our ninety six women without any infection and 15 with abnormal genital tact pathogens. Four women had BV, three GV, four Yeasts, three BHEM, and one had enterococcus infection. We didn't find any woman with Trichomonas Vaginalis, Chlamydia, Ureoplasma or Mycoplasma infection. The group of these 15 women had a median age of 27.7 (range 34-23) years. Twelve of them were Caucasian and three Afro-Caribbean. The average BMI was 23.8 (range 28.4-18.5). Three of these women had a previous ectopic pregnancy and one had two previous ectopic pregnancies. Four women had a previous miscarriage and one had two previous miscarriages. Four women had a previous term delivery and one had two previous term deliveries (Table 6.1).

Table 6.1 Summary of patient characteristics (n=96)

	Vaginal infection (n= 15)	Normal Flora (n=81)
Age (range)	27.7 (45-20)	30.6 (48-19)
BMI (range)	23.8 (28.4-18.5)	25.3 (45-21)
Ethnicity		
Caucasian	12	66
Afro-Caribbean	3	10
Asian		5
Number of women with previous ectopic pregnancies		
one previous ectopic pregnancy	3	15
two previous ectopic pregnancies	1	5
Number of women with previous early miscarriages		
one previous early miscarriage	4	21
two previous early miscarriages	1	9
Number of women with previous term deliveries		
one previous term delivery	4	12
two previous term deliveries	1	6

The group of the 81 women who didn't have any vaginal infection had an average age of 30.6 (range 48-19) years. 66 of them were Caucasian 10 Afro-Caribbean and 5 Asian. The average BMI was 25.3 [range 45-21]. Fifteen of

these women had a previous ectopic pregnancy and five had two previous ectopic pregnancies. Twenty one women had a previous miscarriage and nine had two previous miscarriages. Twelve women had a previous term delivery and six had two previous term deliveries (Table 6.1).

In the cervical mucus samples obtained from the women with abnormal genital tract pathogens the mean of total leukocytes as percentage of total dispersed cells was 15.21%. CD16+ granulocyte were the predominant subpopulation of leukocytes (6.16 % of all the cells). The numbers of CD20+ was 0.24 % of all the cells. CD14+, CD56+ and CD69+ were 0.09%, 0.1% and 0.1% of all the cells respectively. Moreover, there were scanty CD3+,CD4+ and CD8+ in this group.

In the samples from the women who didn't have any vaginal infection the mean of total leukocytes as percentage of total dispersed cells was 17.4%. CD16+ granulocyte were again the predominant subpopulation of leukocytes (8.66% of all the cells). The numbers of CD20+ was 0.42% of all the cells. CD14+, CD56+ and CD69+ were 0.24%, 0.17% and 0.1% of all the cells respectively. Moreover, there were scanty CD3+,CD4+ and CD8+ in this group.

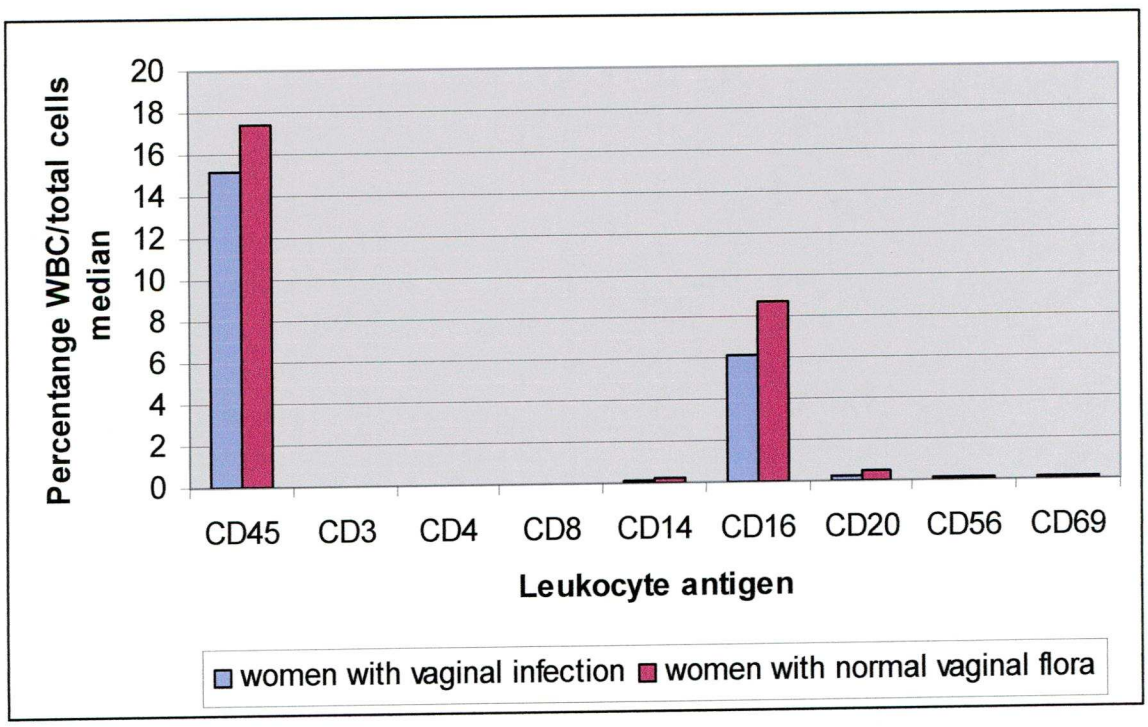
Analyzing these results, we found that there was no significant difference in leukocyte populations between the first and second sample groups. Comparative results between the two groups are shown in Table 6.2 and in chart 5.

Table 6.2 Cervical leukocyte populations for women with and without vaginal infection (n=96).

Percentage WBC/ total dispersed cells median (interquartile range)

Leukocyte antigen	Vaginal infection (n= 15)	Normal Flora (n=81)	P-value
CD45	15.21 (2.4-28.7)	17.4 (3.2-26.8)	0.78
CD3	0 (0-0.24)	0 (0-0.30)	0.86
CD4	0 (0-0.21)	0 (0-0.25)	0.79
CD8	0 (0-0.14)	0 (0-0.18)	0.67
CD14	0.09 (0-0.16)	0.24 (0-0.34)	0.23
CD16	6.16 (1.5-15.3)	8.66 (0.1-14.6)	0.65
CD20	0.24 (0.1-3.4)	0.42 (0.09-4.5)	0.58
CD56	0.10 (0-5.6)	0.17(0-3.4)	0.72
CD69	0.1(0-0.3)	0.1(0-0.3)	0.76

Chart 5: Cervical leukocyte populations for women with and without vaginal infection



6.5. Discussion

By the time a woman is admitted in preterm labor there may be irreversible changes in the cervix that render attempts to inhibit the process unsuccessful. Although infection is an important factor in inducing preterm labour and delivery (Pararas et al., 2006; Galask et al., 1984; Romero et al., 2006)., the use of antibiotics for the prevention of preterm birth causes confusion.

Many studies found a significant reduction in preterm delivery when metronidazole, was used to treat abnormal vaginal flora in high risk women alone (MacDonald et al., 1997; Morales et al., 1994), or in combination with erythromycin (Hauth et al., 1995).

However, they are at variance with Carey et al., (2000) who found that although treatment with metronidazole reduced the rate of bacterial vaginosis, there was no effect on the occurrence of preterm delivery or other adverse perinatal outcomes. Moreover, Hauth et al., (2001) reported that mid-trimester treatment with metronidazole and azithromycin of women with a history of a previous preterm birth, bacterial vaginosis and a positive fetal fibronectin in cervical/vaginal fluid did not reduce the rate of preterm delivery in patients less than 35 and 32 weeks of gestation. In addition, a large randomized single-blind trial in which women with trichomoniasis were randomly allocated to metronidazole or placebo concluded that treatment of asymptomatic women did not prevent the rate of preterm delivery (Klebanoff et al., 2001). Metronidazole has also failed to show any benefit in reducing preterm birth in a number of randomised controlled trials in women screened from a general antenatal population, usually by abnormal vaginal flora (Hillier et al., 1988; Klebanoff et al., 2001). Besides, Eschenbach et al., (1991) did not demonstrate benefits on pregnancy outcome treating Ureaplasma urealyticum with erythromycin.

The ORACLE trial confirmed that systematic antibiotic administration for all women with spontaneous preterm labour provided no benefits (Kenyon et al., 2001b).

Furthermore, Shennan et al., (2006) demonstrated that metronidazole is not only ineffective in preventing preterm delivery in women designated at high risk for preterm delivery, but moreover maybe detrimental, possibly leading to a greater incidence of preterm delivery.

Analysis of results of other groups agrees with this bizarre observation. An increased risk of delivery has been demonstrated in association with metronidazole treatment, compared with placebo, in women who had a history of preterm birth or mid-trimester pregnancy loss (Andrews et al., 2003; Odendaal., 2002).

There is no obvious explanation for these discrepancies between the former and later trials on the effect of antibiotics on the pregnancy outcome. One reason that may explain the absence of benefit by antibiotics is that 'dying' microorganisms may result in an inflammatory response that increases the risk of delivery (Klebanoff et al., 2001).

Of course, the exact role of infection in the pathophysiology of preterm birth is still unclear. The bacteriostatic properties of antibiotics could theoretically have an adverse influence on the normal vaginal flora, allowing opportunistic organisms to colonise. Hydrogen peroxide producing lactobacilli are protective against infective morbidity and therefore metronidazole may influence the lactobacilli profile (Wilks et al., 2004).

There is also increasing evidence to propose that there may be a hereditary component to risk of preterm delivery, which includes increased frequency of genetic polymorphisms in the mother which may predispose towards increased susceptibility to infection or an inflammatory response to infection (Romero R, Chaiworapongsa T et al., 2004). Individuals differ in their ability to mount an inflammatory response. In the context of the vaginal ecosystem,

Simhan et al., (2003) have proposed that many "hyporesponsive" mothers may not be able to control microbial burden, and this in turn predisposes to ascending intrauterine infection and preterm labour. In contrast, "hyperresponder" mothers have an excessive local inflammatory response, which can lead to tissue damage and increased risk of preterm labour. Thus, both microbial exposure and host response in the lower genital tract play important role in the pathogenesis of preterm labour.

In view of this data we tried to investigate the cervical leucocyte response to abnormal genital tract pathogens. Ninety six pregnant women with a history of previous preterm labour had their cervical leukocyte population evaluated using immunohistochemistry. In addition, they were screened for eight genital tract infections. Culture for aerobic and anaerobic bacteria and polymerase chain reaction analyses for Ureaplasma urealyticum, Chlamydia and Mycoplasma hominis were performed, in order to identify any abnormal genital tact pathogen. 15 women had vaginal infection: Four women had BV, three GV, four Yeasts, three BHEM, and one had enterococcus infection. 81 women had normal vaginal flora. The percentage of cervical leukocytes in women without any vaginal infection was, on average, 17.4% of the total number of cervical cells isolated. In the cervical mucus samples obtained from the women with abnormal genital tract pathogens the mean of total leukocytes as percentage of total dispersed cells was 15.21%. There was no statistically significant difference in the percentage of CD45+ cells in those women with or without inflammation in the vaginal swabs.

Using our quantitative method, we were able to evaluate the leukocyte sub-population in both groups and we found no statistically significant difference. Our findings suggest that leukocytes are present in cervical mucus of all women regardless of the presence of infection. Our finding is in agreement with the results of other studies. Although inflammation often is discussed in conjunction with sexual transmitted disease (STD) (Landers et al., 1990; Given, 1987), authors of recent articles note that the relationship between inflammation and STDs is not conclusive (Dimian et al., 1992; Bertolina et al.,

1992; Ecert et al., 1995). Given the restricted number of subjects in this study, the option that a small difference might exist in leukocyte counts between those women with a diagnosis and those without a diagnosis of infection cannot be ruled out absolutely.

However, our data showing that a baseline level of leukocytes is present in all cervical samples obtained from pregnant women with a history of previous preterm delivery, which is unrelated to any infection.

Chapter 7

Conclusions

7.1 Introduction

7.2 Final Conclusions

7.3 Future Considerations

Chapter 7

CONCLUSIONS

7.1 Introduction

The 24-36 weeks of pregnancy is a unique gestational time period, falling between the first six months, at which time any pregnancy loss is considered as miscarriage and predating 36 weeks at which time the delivery becomes "term".

The loss of any pregnancy can be devastating to a couple, regardless of gestation. Delivery in the third trimester (24-36 weeks) can be particularly upsetting, as the fetal anatomy will have been checked in details on anomaly scan (18-22 weeks), however the baby could be too premature to survive if delivery occurs.

To highlight the importance of focused managment of women who have increased risk to give birth preterm, preterm labour is included in the Royal College of Obstetricians and Gynaecologists Subspecialty training syllabus of maternal-Fetal Medicine. In addition, many hospitals have established specialized clinics which deal with this problem.
In our study one hundred and six women were recruited from the busy Preterm Labour clinic of the Liverpool Women's NHS Foundation Trust. Using the cytobrush technique we evaluated for the first time the cervical leukocytes population in pregnant women with history of previous preterm labour.

7.2 Final Conclusions

The leukocytes obtained by the cervical cytobrush are mainly derived from the intraepithelial compartment (Prakash M, Kapembwa M et al.,2001). Our study showed that the population of leukocytes compared to epithelium cells, varied significantly between patients. In agreement with our observation, older studies showed the same results on non pregnant women regardless of the presence of demonstrable infection and regardless of the diagnosis of cervicitis (Judy et al., 1998).

CD16+ granulocyte was the most common sub-population in our high risk pregnant women. Sakamoto et al., (2005) and Bokstrom et al., (1997) had different results when they studied cervical biopsies taken from pregnant women. For this reason and as there is not yet any other investigation on the epithelial granulocyte expression, we propose that granulocytes are actively secreted into the cervical mucus leading to an increased population of granulocytes in the epithelial cervical region. This is supported by the finding of high numbers of granulocytes in the cervical mucus of both pregnant and non-pregnant women obtained by swabs (Luo et al., 2000).

Our findings on the proportions of leukocytes that were macrophages and B cells are similar to that previously reported in the sub-epithelial and stromal cervical regions (Bokstrom et al., 1997; Sakamoto et al., 2005). It looks to be fact that B-lymphocytes are few in number and are found only in lymphoid aggregates (Johansson et al., 1999).

Our observation of low numbers of T cells among the leukocytes is similar to that reported in the non-pregnant cervix (Prakash M, Kapembwa M et al., 2001). This is in contrast to previous publications regarding cervical leukocytes in pregnancy. (Bokstrom et al., 1997; Sakamoto et al.,2005).

Hence, our finding of a lack of T cells in the cervical epithelium suggests that T cells are not secreted into the cervical mucus in pregnancy.

Our results also indicate that the CD16+ granulocytes were the predominant subpopulation of leukocytes both in early and late pregnancy and in addition, as the gestational age increases, there is no significant difference in leukocyte populations. These findings agree with previous studies (Knudsen et al., 1997; Timmons and Mahendroo 2006) and disagree with others (Bokstrom et al., 1997; Junqueira et al., 1980; Ludmiret et al., 2000). As our study is the first which examine the cervical leukocytes in a high risk population, the disagreement of some of our findings with the results of others studies, could reflect the difference in the mechanisms involved in cervical ripening in pregnant women with history of PPTL, from those in pregnant women who don't have such antenatal history. Moreover, we should note that we have examined the leukocyte population in the first and second trimester of pregnancy and not during the intrapartum period, something that most studies have done.

When we examined prospectively the relation between the length and dilatation of the cervix and the cervical subtypes of leukocytes in our population, we found that cervical leukocytosis was not a prologue to cervical shortening and funnelling. CD16+ granulocytes were the predominant subpopulation of leukocytes both in women who subsequently developed either of these cervical changes and in those who did not. Additionally, there were no difference in leukocyte populations in women who subsequently developed either of these cervical changes compared to those who did not.
When we investigated whether cervical epithelial leukocytes were related to pregnancy outcome, we did not find the elevated number of granulocytes that would be expected if these women went into recurrent preterm labour because of an excessive host immune response to genital tract flora early in pregnancy. (Kenyon et al., 2001a; Romero et al., 2002; Winkler, 2003).

An explanation of this finding could be our inability to ascertain if any of our women had GP visits and our lack of knowledge about specific treatments

that they could have. In agreement with this explanation, there was low rate of infection among our population of women. In addition, our second cervical sample occurred 1–3 months prior to labour; thus, it is possible that the sampling was too early to detect an increase in cervical mucus leukocytes close to preterm labour.

We can also suppose that the leukocyte infiltration of the cervix may happen after the start of labour and may be a result of labour and not a cause of it. This hypothesis is in keeping with the findings of many study groups, such as Sakamoto et al., (2005), Osman et al., (2003), Timmons and Mahendroo, (2006).

Very interesting was our finding that a specific leukocyte subtype, the macrophage (CD14+), was less common in the cervical epithelium among high risk women who subsequently had a recurrent spontaneous preterm delivery compared to those that had a term delivery in our group of study.

Simhan et al., 2003 found that women with low concentrations IL-1 b, IL-6, and IL-8 in the vaginal fluid are more likely to subsequently develop clinical chorioamnionitis. Our study takes this concept further: as cytokines are produced primarily by leukocytes (Young et al., 2002), limited number of macrophages may result in low concentrations of cytokines and this in turn may result in susceptibility to vaginal organisms, ascending infection, choriamnionitis and preterm delivery.
Based on this, we can say that cervical epithelial macrophages may serve to prevent recurrent preterm labour, possibly by preventing ascending infection.

Finally, our data showed that a baseline level of leukocytes is present in all cervical samples obtained from pregnant women with a history of previous preterm delivery, regardless of the presence of infection. Our finding is in agreement with the results of other studies. (Dimian et al., 1992; Bertolina et al., 1992; Ecert et al., 1995).

7.3 Future Considerations

The study undertaken for this thesis has involved patient interrogation and database creation, laboratory investigation, attendance of specific clinical practice, patient counselling and systematic search of the literature.

Accurate diagnosis of pre-term labour can allow for the avoidance or delay of pre-term birth and where this is not possible, earlier provision can be made to provide optimal support for the immature infant. Many tests have been proposed to predict spontaneous preterm birth. However, the pathogenesis of the syndrome is currently poorly understood and the prediction of PTL is tricky.

We hope that our study has helped towards this end. Of course there is much work to be done. There is no doubt that more clues to the understating and prediction of PTL will be provided as improved technology and advanced laboratory science will be applied in this field.

Appendix

Appendix 1: APAAP- Materials required

TBS buffer

Primary antibodies

BSA (bovine serum albumin)

Normal human serum, heat inactivated

Rabbit anti-mouse Ig (DAKO code Z 259)

APAAP complex (DAKO code D 651)

Alkaline phosphatase substrate

Fast red TR salt (Sigma F-8764)

PAGG (100mM stock: 27mg/ml, Sigma P-3501, store aliquoted -20°C)

Haemalum stain (BDH 350604T, filtered)

0.45µm filter

Humidified slide box

Dako pen

Acetone and/or methanol

Appendix 2: Buffers

TBS (test buffer saline):

Tris 50mM	**6g/l**
NaCl 150mM	**8.7g/l**

Adjust to pH 7.6 using HCl

To prepare 5 litres: 30g Tris & 43.5g NaCl

Alkaline phosphatase substrate:

Prepare stock solution in glass bottle, store 4°C

Napthol AX-MX Phosphate (Sigma F-1500)	**10mg**
Dimethyl formamide (Sigma D-4254)	**1.0ml**
Levamisole 1.0M (Sigma L-9756), stored-20°C	**50µl**
0.1M Tris buffer pH 8.2	**50mls**

Appendix 3: Immunohistochemistry- APAAP (ALKALINE PHOSPHATASE-ANTI-ALKALINE PHOSPHATASE)

Method

1. **Remove slides from freezer and allow to reach room temperature.**
2. **Mark areas to be stained with a 'Dako' pen.**
3. **Fix slides: sections in acetone, 10 mins.**
   cvtospins in acetone:methanol (50/50), 90 sees.
4. **Rinse TBS. wash 5 minutes TBS. Do not allow to drv out. Place in humidified chamber.**
5. **Dilute antibodies in TBS + 0.5% BSA. 50ul per sample, incubate 30 minutes room temperature.**
6. **Rinse TBS, wash 5 minutes in TBS. Repeat.**
7. **Dilute Rabbit anti-mouse Ig (or appropriate 2nd antibody) 1:25 in TBS + 5% normal human serum. 50ul per sample, incubate 30 minutes room temperature.**
8. **Rinse TBS. wash 5 minutes in TBS. Repeat.**
9. **Dilute APAAP complex 1:50 in TBS. 50ui per sample, incubate 30 minutes room temperature.**
10. **Rinse TBS. Wash 5 minutes in TBS. Repeat.**
11. **Weigh 10mg Fast red TR salt into a glass bottle. Add 1Omls alkaline phosphate substrate. Filter through 0.45um filter into a second glass bottle.**
12. **Require 50ul per sample, prepare 100mM PAGG. 10ul per ml substrate solution.**
13. **Incubate 50ul per sample. 20 mins room temperature.**
14. **Wash once TBS. Once with distilled water.**
15. **Counterstain with Haemalum. Time varies but usuailv 30 seconds.**
16. **Wash well with tap water.**

17. Air dry room temperature.

18. Mount in aquamount.

Appendix 4: COUNTING CELLS USING A HAEMOCYTOMETER

The haemocytometer is a specialized microscope slide used to count cells. It has a thick base and uses a special cover glass which is thick enough to stay flat under the pull of surface tension from the solution in the counting chamber.
The haemocytometer has two polished surfaces, each of which displays a precisely ruled, sub-divided grid. The grid consists of nine large primary squares each of these large squares has an area of $1mm^2$. The squares are bordered by three closely spaced lines which are used to determine if cells lie within or outside the grid. The large central square is further divided into 25 medium squares which, in their turn, are sub-divided in 16 small squares. Also the four large corner squares are subdivided into 16 squares. When the cover slip is pressed down over the grid so that the interference patterns appear, the depth of the chamber is 0.1mm (appendix 5).

The total volume over each large square is therefore:
$1 \times 1 \times 0.1 = 0.1 mm^3$
$= 0.0001 cm^3$
$= 10^{-4}$ ml

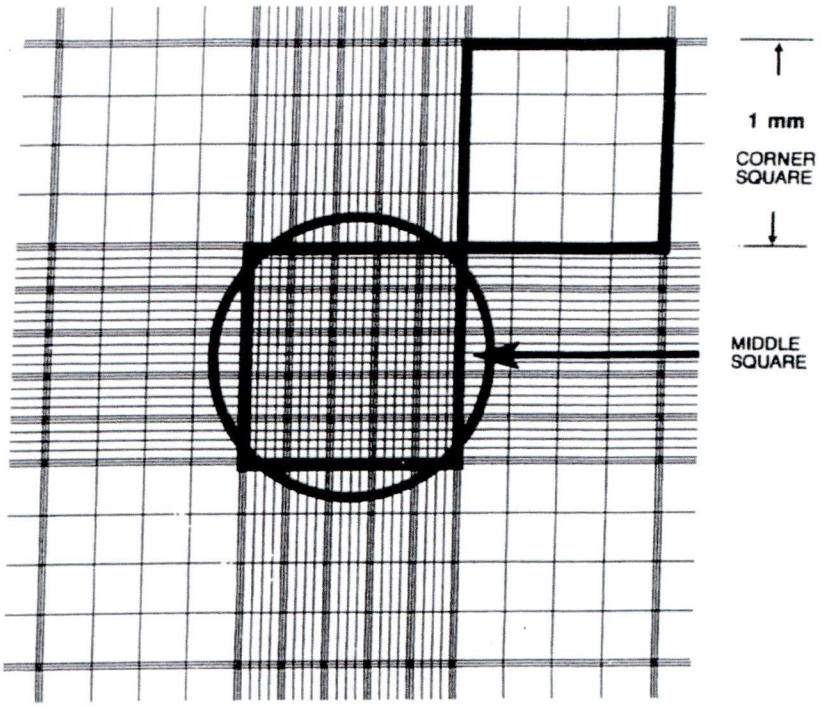
To perform a count a small amount of the suspension is taken into a Pasteur pipette and used to fill the well of the haemocytometer. Count the cells in the large centre square and the four large corner squares. Cells touching the upper and right hand perimeter lines should be ignored; those touching the lower and left hand perimeter lines should be counted.

A raw count of over 100 cells is required for accuracy. If insufficient cells are present then count further squares or repeat the whole procedure. If the cell concentration is high, dilution of the cell suspension may be required. The cell density is calculated, remembering to allow for any dilutions that may have been made.

$$\frac{\#\text{ cells} \times (\text{dilution factor})}{\#\text{ 1mm squares counted}} \times (1 \times 10^4) = \#\text{cells/ml}$$

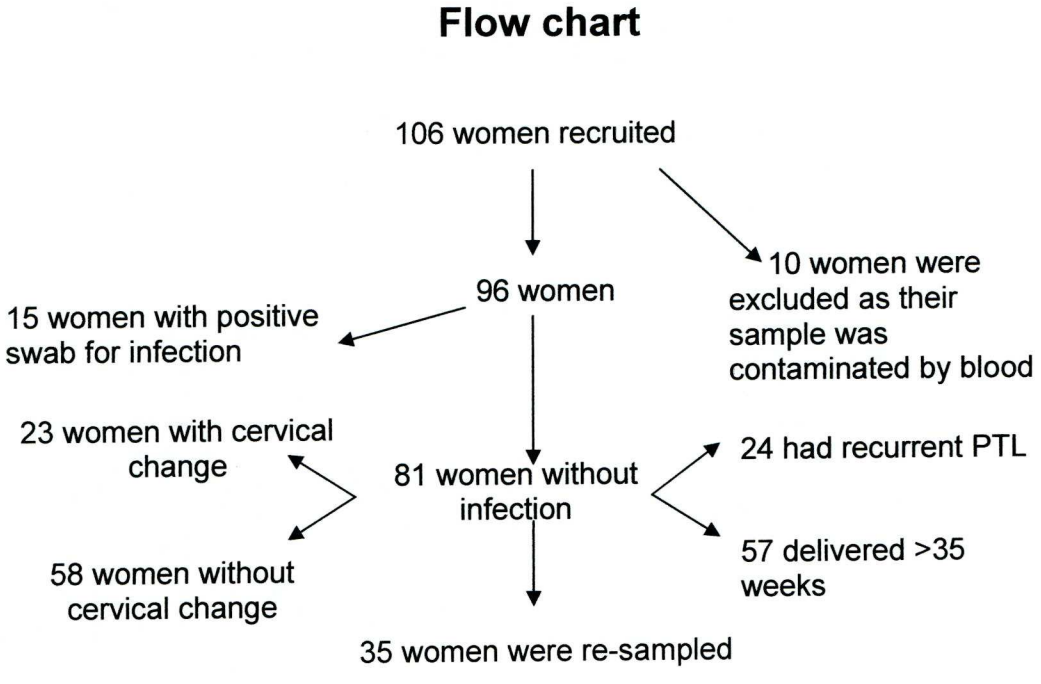
cells/ml x #mls of cell suspension=Total#cells

Appendix 5: STANDARD HEMOCYTOMETER CHAMBER



The circle indicates the approximate area covered at 100 x microscope magnification (10 x ocular and 10 x objective) Include cells on top and left touching middle line. Do not count cells touching middle line at bottom and right. Count 4 corner squares and middle square in both chambers (one chamber represented here).

Appendix 6: Flow chart of the study



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PUBLICATIONS ARISING FROM THE STUDY

Whitworth MK, **Pafilis I**., Vince G., Quenby S. (2007) Cervical leukocyte sub-populations in idiopathic preterm labour. Journal of Reproductive Immunology, 75, 48–55.

Pafilis I,Nickson P,Quenby Q,Vince G. (2005) Variation in the cervical lecucoyte response to vaginal flora in women at high risk of preterm labour (abstract). BJOG,112,(4),511.

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Journal of Reproductive Immunology 75 (2007) 48–55

JOURNAL OF REPRODUCTIVE IMMUNOLOGY

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Cervical leukocyte sub-populations in idiopathic preterm labour

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Received 11 August 2006; received in revised form 23 November 2006; accepted 21 December 2006

Abstract

Objective: To investigate cervical epithelial leukocyte sub-populations in pregnant women with a history of idiopathic preterm labour.

Methods: A prospective observational study was undertaken of 106 women with a past history of idiopathic preterm delivery following spontaneous labour. A cytobrush was used to sample the epithelium of the cervix at 12–16 weeks of gestation and again 8 weeks later. All women had investigations for cervical and vaginal infection as well as serial transvaginal ultrasonography of their cervix; the mode and gestation at delivery were recorded. Leukocyte sub-populations were examined using immunocytochemistry, and the number of leukocytes per total cell count was calculated.

Main outcome measures: Cervical epithelial leukocytes populations were (1) described in pregnancy, (2) observed over increasing gestation, (3) analysed in women who developed marked cervical shortening and (4) in those whose preterm labour recurred.

Results: There was no significant change in cervical epithelial leukocyte populations during the second trimester of pregnancy. There was no association between cervical leukocytes and cervical shortening. Women with idiopathic preterm labour that recurred had fewer cervical macrophages at the beginning of the second trimester of pregnancy than those whose subsequent pregnancy progressed beyond 35 weeks of gestation.

Conclusions: Cervical epithelial macrophages may serve to prevent recurrent preterm labour, possibly by preventing ascending infection.

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Keywords: Leukocytes; Preterm labour; Cervix; Leukocyte

1. Introduction

Despite the fact that preterm birth is the leading cause of neonatal mortality and morbidity in the western world, approximately 50% of premature births are the result of spontaneous preterm labour of no known cause (Slattery and Morrison, 2002). A woman who has had a preterm delivery is at increased risk of preterm delivery in subsequent pregnancies (Adams et al., 2000). Spontaneous preterm birth at an early gestational age is more predictive of recurrence and is highly associated with subsequent early spontaneous preterm birth (Mercer et al., 1999). These findings suggest that some women have underlying factors that contribute to recurrent preterm labour.

The exact mechanisms leading to the initiation of preterm labour are not yet known. However, some of the processes involved in preterm labour have been described. Changes in the uterine cervix are thought to

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0165-0378/$ – see front matter © 2007 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.jri.2006.12.004

be an important part of biological adaptation for labour. Prior to the onset of labour, the cervix undergoes a process known as ripening during which it becomes softer and shorter (Olah and Gee, 1992). Cervical ripening occurs prior to dilatation and is a prerequisite for vaginal delivery. Cervical ripening is the process by which the cervix changes from a tightly closed structure designed to maintain an intrauterine pregnancy to a soft, compliant organ capable of dilating to accommodate passage of the fetus. This process involves profound tissue remodelling and is accompanied by disruption of collagen fibrils, alteration in glycosaminoglycans and oedema of the cervical stroma (Junqueira et al., 1980; Osmers et al., 1995). It has been postulated that the process of cervical ripening and dilatation resembles an inflammatory response (Liggins, 1981; Yellon et al., 2003). Under this paradigm leukocytes are thought to migrate into the cervix before labour (Luo et al., 2000; Osman et al., 2003). This cervical leukocyte infiltrate is composed principally of granulocytes and macrophages (Osman et al., 2003) which are thought to secrete inflammatory cytokines (Luo et al., 2000; Winkler, 2003) which, in turn, initiate labour (Kurkinen-Raty et al., 2001; Romero et al., 2002; Keelan et al., 2003; Kishida et al., 2003).

Previous investigators have found an increase in cervical leukocytes during labour compared to women not in labour, and a further increase in cervical leukocytes postpartum (Bokstrom et al., 1997; Knudsen et al., 1997; Luo et al., 2000; Osman et al., 2003; Sakamoto et al., 2005). However, the cervical lymphocyte infiltrate that has been described previously could be a consequence rather than a cause of labour. In support of the former possibility, a mouse model of preterm labour found that the leukocyte infiltrate only occurred during and after labour not prior to the onset of labour (Timmons and Mahendroo, 2006). Hence, the question remains - is cervical leukocyte infiltration a factor in initiating preterm labour rather than an observation occurring after the onset of labour in humans? A problem encountered by previous investigators is that biopsies of the human cervix are associated with a risk of haemorrhage and infection (Bokstrom et al., 1997; Knudsen et al., 1997; Osman et al., 2003; Sakamoto et al., 2005). This meant that previous authors could not sample patients who are at high risk of preterm labour because the biopsy itself could cause inflammation, infection and, subsequently, preterm labour.

In order to test the hypothesis that increases in cervical leukocyte populations are associated with the onset of recurrent idiopathic preterm labour, we adapted a previously described, non-traumatic method of sampling the cervix (Prakash et al., 2001). Prakash et al. (2001) described using a cytobrush to sample the non-pregnant cervix and showed that this method sampled a unique population of leukocytes that was different from that in peripheral blood.

The aim of the present study was to investigate cervical epithelial leukocyte populations in women at high risk of spontaneous idiopathic preterm labour. We have described the cervical epithelial leukocyte population in women with a past history of idiopathic preterm labour, optimised the timing of sampling and then tested the hypothesis that recurrent preterm labour is associated with an excessive cervical epithelial leukocytosis prior to the onset of labour.

2. Methods

2.1. Pilot phase of the study

Local ethical permission was obtained to develop a technique to study cervical leucocytes in pregnancy. After informed consent had been obtained, five women who had a past history of preterm delivery following spontaneous preterm labour and were in the second trimester of pregnancy were included in each group. The two techniques used were:

(a) A cotton swab was rotated 360° in the external os of the cervix.
(b) A fine cervical cytobrush (Cervex-brush, Rovers Medical Devices, Oss, Netherlands) was gently rotated once in the cervical canal.

2.2. Sample processing

Samples were immediately diluted in 5 ml of phosphate-buffered saline (PBS) containing 0.1% bovine serum albumin (BSA). Samples were mixed well to disrupt the mucus and then centrifuged for 10 min at 1200 rpm at room temperature. The pellet was then snap frozen and stored at −80 °C. For use the pellet was re-suspended in 2 ml PBS containing 0.1% BSA and diluted 1:1 in trypan blue for cell counting. Cell number was determined using a haemocytometer (Weber Scientific, Hamilton, USA).

Using the cotton swab, only $(1–5) \times 10^4$ cells per sample were obtained. In contrast, using the cytobrush technique, we obtained substantially more cells, $(4–6) \times 10^6$ per sample, a similar number to that recorded in the previously published study (Prakash et al., 2001). Hence, the cytobrush technique was used for the main study.

2.3. Subjects for the main study

One hundred and six women were recruited from an antenatal clinic dedicated to the care of women with a history of preterm labour at the Liverpool Women's NHS Foundation Trust. The study was approved by the Liverpool Adult Local Research Ethics Committee. Written informed consent was obtained from each subject. The 106 women each had a history of at least one idiopathic preterm delivery following spontaneous labour between 22 and 32 weeks of gestation. We did not include women who experienced preterm labour but did not deliver prior to 32 weeks gestation. Women were excluded from the study if they had a multiple pregnancy or if their previous preterm labour had a recognisable cause, e.g. placental abruption, intrauterine growth restriction or pre-eclampsia, or was iatrogenic. Women were also excluded from the study if they had had a previous cone biopsy. Women whose preterm birth was thought to be contributed to by infection or inflammation were included in this study. In Liverpool Women's Hospital, preterm deliveries with positive microbiology or inflammation detected on placental histology are considered to be idiopathic as the clinician can never be sure whether the infection came before or after the onset of labour.

2.4. Sample collection

At study entry, sterile speculum examination was performed, and swabs were appropriately taken and examined for bacterial vaginosis, trichomonas vaginalis, gonococcus and other pathogenic organisms. Chlamydia antigen, ureaplasma urealyticum and myoplasma hominis were tested for using polymerase chain reaction (PCR). Then, the cytobrush was used to collect cervical cells as described above (b).

Eighty-one women had their cervical leukocyte populations sampled at 12–16 weeks of gestation; of these, 35 women had their cervix sampled twice at 8-week intervals. As suggested by Prakash et al. (2001), samples were excluded if contaminated by blood ($n = 10$). Women were not included if they had received antibiotics in pregnancy prior to their appointment. Samples were also excluded if a pathogenic genital tract infection was detected on the swabs or if they had bacterial vaginosis and were subsequently given antibiotics ($n = 15$).

The severity of the patients' past obstetric history, shown in Table 1, highlights the significance of any recurrence of their preterm labour. In total, 48 (59%) women had a previous delivery prior to 28 weeks of gestation, 38 (47%) had suffered at least one neonatal death due to prematurity and two (3%) had children that survived premature birth but were affected by cerebral palsy (Table 1). No woman in the study was underweight, and only two women were obese. The majority were Caucasian, and all women had sufficient education to read the patient information leaflet and sign their name on the consent form (Table 1).

2.5. Immunohistochemistry

The cell suspension described previously for cell counting (sample processing) was diluted to a

Table 1
Summary of patient characteristics at study entry

	Recurrent preterm delivery ($n = 24$)	Delivered after 35 weeks gestation ($n = 57$)	P-Value
Age (mean years)	30.2	31	NS
BMI (mean)	26	25	NS
Range	(21–40)	(21–45)	
Number underweight, BMI < 20	0	0	
Number obese, BMI > 35	1	1	
Ethnicity			
Caucasian	21	45	NS
Afro-Caribbean	2	8	
Asian	1	4	
Mean number of previous preterm deliveries after spontaneous labour	1.7	1.2	NS
Number of patients who had a previous birth before 28 weeks' (%)	14 (58%)	34 (60%)	NS
Number of patients who had a previous neonatal death due to prematurity (%)	10 (41%)	28 (49%)	NS
Number of patients with an existing child with cerebral palsy due to prematurity (%)	2 (8%)	0 (0%)	NS

concentration of 1×10^6 cells/ml. For immunohistochemistry, 50 μl of cell suspension was placed onto each glycerine-albumin coated teflon multi-well slide and air-dried overnight at room temperature. Slides were stored at −20 °C prior to use. Immunohistochemistry was performed using an alkaline phosphatase anti-alkaline phosphatase (APAAP) method (Serotec Ltd, Kidlington, Oxford, UK). Primary antibodies are detailed in Table 2. The pre-prepared slides were fixed in acetone for 10 min at room temperature. Cells were rehydrated in 0.05 M Tris-buffered 0.15 M saline (TBS), pH 7.6 for 10 min and then incubated with appropriately diluted primary monoclonal antibody (mAb) in TBS. Slides were washed three times in TBS and incubated with rabbit anti-mouse secondary antibody. Slides were washed again in TBS, and overlain with mouse monoclonal APAAP for 30 min. Bound primary mAbs were detected by incubation with Fast Red (Sigma, Dorset, UK). Cell smears were mounted in Aquamount (BDH, Poole, UK). Controls were performed for all samples and sections were incubated with normal IgG instead of primary mAb.

2.6. Quantification of cells

All cells were counted in 10 fields of 400× magnification using a 10 mm × 10 mm graticule covering an area of 0.0625 mm^2 by an observer blind to the origin of the sample. Positive cells, identified by the presence of red staining, were also counted in 10 fields at 400× magnification. Leukocyte sub-populations were expressed as a fraction of the total cell count to account for inter-patient variation in the numbers of cells collected and the amount of cervical epithelium sampled.

2.7. Preterm labour clinic protocol

As per clinic protocol, all women had serial cervical length measurement using trans-vaginal ultrasonography at least at 12, 16, 20, 24 and 28 weeks of gestation. The ultrasound examination was performed in a standardised manner with a stress test of the cervix (Shennan and Jones, 2004). For a stress test, mild abdominal pressure was applied for 30 s whilst contemporaneously scanning the cervix. A positive test was one in which cervical shortening and funnelling was produced (Shennan and Jones, 2004). Women with significant cervical shortening to a length <25 mm were managed with bed rest.

2.8. Data and statistical analysis

Statistical analysis was performed with Stats Direct software (Cheshire, UK). Differences in the number of leukocytes sub-type per total cells between two groups were analysed using Mann–Whitney's *U*-test. A p-value 0.05 was considered significant.

3. Results

Using the cytobrush technique, a similar proportion of the total cell population including epithelial cells were leukocytes compared to that reported in the previous publication (approximately 10% in both studies) (Table 3), confirming our ability to reproduce the technique (Prakash et al., 2001).

The most common leukocytes present were positive for CD16 (Table 3; Fig. 1a). No staining was observed in negative controls with mouse IgG (Fig. 1b). Antibodies to CD16 stain both granulocytes and NK

Table 2
Primary monoclonal antibodies

Monoclonal antibody	Specificity	Source	Clone	Dilution
CD45	All haematopoetic cells	Culture supernatant, American Type Culture Collection	F10894	Neat
CD3	T lymphocytes	Serotec	MCA 463	1:100
CD4	T (helper) cells	Dako	M0716	1:50
CD8	T (cytotoxic) cells	Dako	M0707	1:50
CD14	Macrophages	Culture supernatant, American Type Culture Collection	3C10	Neat
CD16	Natural killer cells, T subset, macrophages, granulocytes	Pharmingen	30621A	1:100
CD20	B lymphocytes	Immunotech	1925	1:50
CD56	Natural killer cells, T subset	Serotec	MCA591	1:50
CD69	Activated T, B, NK cells and macrophages	Serotec	MCA1442	1:100
Mouse IgG	Negative control	Serotec	MCA928	1:100

Table 3
Serial first and second trimester leukocyte populations

Leukocyte antigen	Percentage leukocytes/total cells median (inter quartile range), $n = 35$		*P*-Value
	First sample 12–16 weeks of gestation	Second sample 20–24 weeks of gestation	
CD45	11.6 (4.85–21.9)	7.4 (2.8–34.1)	NS
CD3	0.1 (0–0.5)	0.1 (0–0.5)	NS
CD4	0 (0–0.3)	0 (0–0.3)	NS
CD8	0 (0–0.3)	0 (0–0.2)	NS
CD14	0.2 (0–1.2)	0.1 (0.1–0.3)	NS
CD16	5.1 (1.2–9)	3.1 (0.4–14.8)	NS
CD20	0.35 (0.1–0.3)	0.50 (0.1–0.7)	NS
CD56	0.2 (0–1)	0.1 (0–0.7)	NS
CD69	0.1 (0–0.3)	0.1 (0–0.3)	NS

cells. As antibodies to CD56 also stain NK cells and, because there were minimal number of CD56+ cells, we can assume that anti-CD16 stained granulocytes (Table 3). Thus, the dominant leukocyte detected in this study was the granulocyte. The second most prevalent leukocyte was CD20+ B cells (Table 3). CD14-positive macrophages were detected in some samples (Table 3). There were very few T cells or activated leukocytes (Table 3).

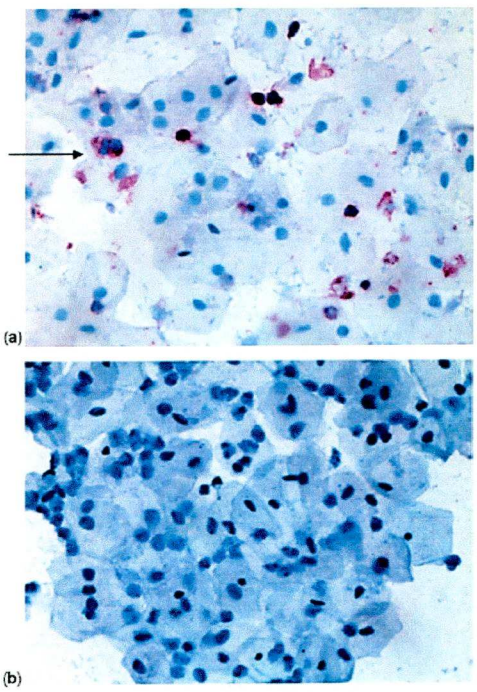

Fig. 1. Cytobrush cell smear stained for CD16 (a) and negative control (b). Epithelial cells and granulocytes → can be seen.

There was no significant difference in leukocyte populations between the first and second samples (Table 3). As some women delivered prior to the second sampling, the first sample (12–16 weeks of gestation) was determined to be the optimal time for the prospective study.

3.1. Prospective study

Serial transvaginal ultrasonography until 28 weeks of gestation identified 23 women who had a positive stress test (cervical funnelling upon application of fundal pressure) and nine who developed cervical shortening <25 mm. There were no differences in leukocyte populations in women who subsequently developed either of these cervical changes compared to those who did not (data not shown).

In the study pregnancy, 24 women went into spontaneous preterm labour and delivered prior to 35 weeks of gestation (range 22–34 weeks gestation), but only four of these delivered prior to 28 weeks of gestation. Fifty-seven women delivered after 35 weeks of gestation. There was no difference in the demographics of those whose preterm labour and delivery recurred and those who delivered later (Table 1).

The median numbers of the major cervical leukocyte populations in women with recurrent spontaneous preterm labour and delivery prior to 35 weeks gestation, and those delivering after 35 weeks of gestation, are shown in Table 4. There were significantly fewer CD14+ macrophages in the first samples taken from those patients who subsequently had recurrence of their preterm labour and delivered prior to 35 weeks of gestation ($p < 0.01$) (Table 4).

Table 4
First trimester cervical leukocyte populations for women with recurrent and non-recurrent preterm labour

Leukocyte antigen	Percentage leukocytes/total cell median (inter quartile range)		*P*-Value
	Recurrent preterm labour ($n = 24$)	Delivered >35 weeks of gestation ($n = 57$)	
CD45	7.4 (3.17–20.14)	13.92 (4.68–29.35)	NS
CD3	0 (0–0.18)	0 (0–0.16)	NS
CD4	0 (0–0.19)	0 (0–0.30)	NS
CD8	0 (0–0.03)	0 (0–0.27)	NS
CD14	0 (0–0.20)	0.26 (0.1–0.5)	0.008
CD16	2.29 (0.59–9.03)	6.08 (1.10–13.93)	NS
CD20	0.54 (0.10– 3.54)	0.15 (0–0.15)	NS
CD56	0 (0–0.56)	0.19 (0–1.40)	NS
CD69	0 (0–0.11)	0 (0–0.11)	NS

4. Discussion

This study has comprehensively characterised cervical epithelial leukocytes sub-populations in pregnant women. We found the most prevalent cervical epithelial leukocyte was the granulocyte. Granulocytes were not examined by Prakash et al. (2001) in the non-pregnant cervix, but they were the most prevalent leukocyte subtype in non-pregnant, non-infected women in our unit (unpublished data). Granulocytes were present in low numbers in the sub-epithelial and stromal regions in cervical biopsies taken in pregnancy (Bokstrom et al., 1997; Sakamoto et al., 2005); however, to date, no investigators have examined epithelial granulocyte expression. Our finding of high numbers of granulocytes in the epithelial cervical region suggests that granulocytes are actively secreted into the cervical mucus. This is supported by the finding of high numbers of granulocytes in the cervical mucus of both pregnant and non-pregnant women (Luo et al., 2000).

The finding of low numbers of T cells among the leukocytes is similar to that reported in the non-pregnant cervix. This distinguishes the cervical epithelial leukocyte populations from peripheral blood where there are significant numbers of T cells (Prakash et al., 2001). Our observation is in contrast to previous publications regarding cervical leukocytes in pregnancy which describe a predominance of T cells in the stromal and, particularly, in the sub-epithelial compartment of the pregnant cervix (Bokstrom et al., 1997; Sakamoto et al., 2005). Hence, our finding of a lack of T cells in the cervical epithelium suggests that T cells are not secreted into the cervical mucus in pregnancy.

The proportions of leukocytes that were macrophages and B cells are similar to that previously reported in the sub-epithelial and stromal cervical regions (Bokstrom et al., 1997; Sakamoto et al., 2005).

The cytobrush technique was successfully translated into our population of women at high risk of recurrent preterm labour, thus enabling us to examine the cervical epithelial leukocyte populations prospectively investigate whether cervical epithelial leukocytes were related to pregnancy outcome. The present study found that a specific subset of leukocyte, the macrophage, was less common in the cervical epithelium in the early second trimester of pregnancy among high risk women who subsequently had a recurrent spontaneous preterm labour and delivery compared to those that delivered after 35 weeks of gestation. The women in this project had very poor past obstetric histories, as 59% of women had a previous delivery prior to 28 weeks of gestation, 47% of women had lost one previous baby because of premature delivery and 3% had a child with cerebral palsy due to prematurity. Hence, despite the fact that only four women delivered before 28 weeks of gestation, any recurrence of their preterm labour had the potential to cause significant morbidity and mortality.

Whilst the importance of preventing the recurrence of preterm labour is recognised, predicting which women are at risk of recurrence and developing therapeutic strategies to prevent recurrence have proved difficult (Kenyon et al., 2001; Lockwood, 2002; Terzidou and Bennett, 2002; Kiss and Petricevic, 2004). A widely accepted explanation for preterm labour is that, in susceptible individuals, ascending genital tract infection stimulates a host immune response that, in turn, initiates labour partly due to a bystander effect (Kenyon et al., 2001; Romero et al., 2002; Winkler, 2003). Our study was designed to produce evidence to support this paradigm. Previous authors have described an increase in cervical mucus granulocytes prior to the onset of term labour (Luo et al., 2000). We did not find the high number of granulocytes that would be expected if these women went into recurrent preterm labour because of an

excessive host response to genital tract flora early in pregnancy. However, in our study the sampling occurred 1–3 months prior to labour; thus, it is possible that sampling was too early to detect an increase in cervical mucus leukocytes close to preterm labour. This argument is supported by the relatively low rate of infection among our population of women at high risk of spontaneous preterm labour (only 15 of 105). It is our experience that women with a history of premature delivery will seek medical attention regarding vaginal discharge in the postnatal period or prior to conceiving. It is therefore likely that women had been screened for common genital tract infections prior to pregnancy. This could lead to a reduction in the vaginal bacterial load in early pregnancy and hence to reduced numbers of macrophages in cervical epithelium. It is possible that women with preterm labour develop abnormal vaginal flora later in pregnancy and an inflammatory response which includes high numbers of leukocytes in the cervical epithelium.

However, a number of observations suggest an alternative explanation. Firstly, our findings are consistent with those of Timmons and Mahendroo (2006) who used immunohistochemistry to examine murine cervices both during pregnancy and during and after parturition. Leucocytosis was observed only after labour had commenced and did not precede labour (Timmons and Mahendroo, 2006). Secondly, our findings agree with previous studies that found an increase in macrophages and the chemokine IL-8 after labour has started (Osman et al., 2003; Sakamoto et al., 2005). Thirdly, there is recent evidence that inability to mount an adequate immune response may play as important a role in the development of disease as over-expression of immune mediators (Marks et al., 2006). Crohn's disease is a chronic inflammatory disorder with features which are similar to those produced by infection with organisms such as mycobacteria. In a recent study Marks et al. (2006) showed that, in patients with Crohn's disease, trauma to rectum, ileum or skin led to an abnormally low neutrophil accumulation and lower production of pro-inflammatory IL-8 (Marks et al., 2006). They concluded that, in Crohn's disease, a constitutionally weak immune response predisposes to accumulation of intestinal contents that breach the mucosal barrier of the bowel wall, resulting in granuloma formation and chronic inflammation. We speculate that a similar mechanism is at play in the case of preterm labour with cervical mucus acting as a reservoir for cells important in preventing ascending infection.

Some epithelial cervical leukocytes are secreted into the cervical mucus, and are hence a first line host response to the ascending genital tract infection that has been associated with preterm labour (Kenyon et al., 2001; Kurkinen-Raty et al., 2001; Lockwood, 2002; Romero et al., 2002; Kiss and Petricevic, 2004). Hence, an alternative paradigm to explain our data is that cervical epithelial leukocytes may prevent preterm labour by preventing ascending infection. The role of the cervix as a barrier to infection has been highlighted by recent reports where an increase in microbial invasion into the amniotic cavity was found in women with short cervices (Gomez et al., 2005; Hassan et al., 2006).

There is considerable controversy as to the role of cervical cerclage in the management of women with a past history of preterm labour that has been recently reviewed (Romero et al., 2006). Romero et al. (2006) have described a few subgroups of women for whom cerclage may be beneficial. Sakai et al. (2006) have suggested that the sub-group of women for whom cervical cerclage is beneficial are those with normal cervical mucus IL-8 levels, as cerclage does not prevent preterm labour in women whose membranes are already inflamed as identified by elevated cervical mucus IL-8. The present study takes this concept further. We suggest that a lack of cervical sub-epithelial macrophages is associated with recurrence of preterm labour. Therefore, cervical cerclage could mechanically prevent loss of cervical mucus and its macrophages, and thereby maintain a physical and immunological barrier to ascending infection.

The finding of fewer macrophages in the epithelium cervix of women who had recurrence of their preterm labour suggests further research should be aimed at understanding the cervical defence against infection and possible treatments to improve host defence from local infection.

Acknowledgements

We are grateful to clinical colleagues for referring patients to our clinic, to Lorna Wood for clinical support during the clinic sessions, to Paul Nickson, Michelle Bates and Jo Drury for technical support with immunocytochemistry, and to Mark Turner for checking the manuscript.

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aspirated to determine the true volume (TVol). Data was analysed according to the method of estimation: CUS1 ($B \times H \times L \times 0.52$), CUS2 ($1/6 \times \pi \times B \times H^2 \times 1.23$), CUS3 ($1/6 \times \pi \times B \times H \times L \times 1.25$), mean and maximum DP volume. The TVol correlated better with CUS1 (Spearman's rho = 0.817, $P < 0.001$) and CUS3 ($r = 0.817$, $P < 0.001$), than to CUS2 ($r = 0.746$, $P < 0.001$), DPVmean ($r = 0.525$, $P < 0.001$) and DPVmax ($r = 0.434$, $P = 0.0119$). The mean percentage error using the mean DPVol was 68% (SD = 185, R(−)100–(+)845), and using CUS1 was 4% (SD = 59, R(−)100–(+)237). The DP technique tended to overestimate. We failed to validate the use of DP for the assessment of postpartum urinary residual volume.

Characterisation of human toll-like receptors in the female reproductive tract. D. O. C Anumba, A. Fazeli and C. Brooks. Section of Reproductive & Developmental Medicine-Obstetrics & Gynaecology, University of Sheffield, Jessop Wing, Tree Root Walk, Sheffield, S10 2SF.

Toll-like receptors (TLRs) play a critical role in innate host defence by the recognition of, and signalling response to, distinct microbial components. We sought to localise TLRs 1–6 in the female reproductive tract. Fresh specimens from the fallopian tubes, endometrium, endocervix, ectocervix and vagina of 9 patients following hysterectomy were prepared and stained by immunohistochemistry using antibodies specific for TLRs 1–6.

The vaginal, ecto- and endocervical, endometrial and fallopian tube epithelia stained positively to TLRs 1, 2, 3, 5, and 6. Vaginal and ectocervical epithelia were negative for TLR4 whilst endocervical, endometrial and tubal epithelia were positive. Endocervical glandular cells also showed TLR4-antibody-stained vacuole-like structures that seemed destined for secretion. Blocking peptides specific for the respective antibodies abolished the staining.

TLRs 1–6 are expressed in female reproductive tract epithelium. The unique localisation of TLR4 in the endocervix and upper, but not the lower tract, suggests a key role in host defence to ascending infection.

Cervical stromal impedance measurement in non-pregnant and pregnant women. S. V. Gandhi, S. Mukherjee, P. Milne, B. Brown, D. Walker and D. O. C. Anumba. The University of Sheffield, Jessop Wing, Tree Root Walk, Sheffield, S10 2SF.

Digital assessment of cervical consistency is subjective and of limited value in assessing 'ripening', a process due to increased hydration and collagen degradation. We sought to validate cervical stromal impedance (CSI) measurements using finite element modelling (FEM) and determine normal values in non-pregnant and pregnant states. We compared CSI in 50 non-pregnant and 70 pregnant women using mean CSI at 50–512 KHz and the R parameter of the Cole equation.

Subjects	Median(range) gestation wks	N	Mean (SE) CSI	R*
Nonpregnant	NA	50	1.79 (0.03)	30.99
1st trimester	9.5 (8–11)	20	1.74 (0.05)	28.32
2nd trimester	21 (16–26)	20	1.84 (0.05)	15.73
3rd trimester	39 (37–41)	30	1.89 (0.07)	16.06

* Non-pregnant *vs* 2nd and 3rd trimesters, $P < 0.05$.

Our FEM predicted cervical stromal impedivity *in vivo*. There was a reduction in R in later pregnancy but mean CSI at higher frequencies was not different. Studies of changes associated with ripening are ongoing.

Induction of labour: Can ultrasound assessment of cervix predict the outcome? A. Kulkarni, P. Wardle and V. Akande. Department of Obstetrics & Gynaecology, Southmead Hospital, Westbury-on-Trym, Bristol, BS10 5NB.

80 women having induction of labour were assessed before, and 4–6 hours after, Prostin by transvaginal ultrasound, instead of the Bishop score. For women with a cervical length of <28 mm (n = 39), 36 (92.30%) had a vaginal delivery within 4 to 52 hours. For women with a cervical length of ≥28 mm (n = 41), change in cervical length had a small predictive value for the outcome of IOL. Where length reduced by >15% between the scans (n = 13), 8 (61.53%) delivered vaginally within 13 to 73 hours. When there was <15% or no change in the cervical length (n = 25), 13 (52%) delivered vaginally within 7 to 83 hours. In those with no change or minimal change in the cervical length, other parameters including mid cervical width, internal os dilatation, width of lower segment did not allow any additional prediction of the outcome of labour.

Variation in the cervical leucocyte response to vaginal flora in women at high risk of preterm labour. I. Pafilis, G. Vince, P. Nickson and S. Quenby. University of Liverpool, First floor, Liverpool Women's Hospital, Crown Street, Liverpool, L8 7SS.

Preterm labour (PTL) is the biggest cause of perinatal morbidity and mortality. Genital tract infections are associated with PTL. We aimed to investigate the cervical leukocyte response to vaginal flora in women at high risk of PTL. 25 pregnant women attending the PTL clinic at a tertiary referral hospital had their cervical epithelium sampled with a cytobrush, in the first and second trimester of pregnancy. The women were screened for 8 genital tract infections. Cervical leucocyte populations were examined with immunohistochemistry.

The population of leucocytes compared to epithelial cells varied considerably (0%–56%). The major leucocyte population was CD16+ granulocytes but few T cells or macrophages were present. A significant number of B and NK cells were present in some women. There was no

correlation between the microbiology and leukocyte response. There was a large variation in cervical leucocyte population that did not reflect microbiology findings.

Novel GTPases in human myometrium. J. Lartey, A. Gampel, H. Mellor and A. Lopez Bernal. University of Bristol, CSSB, Division of Obstetrics and Gynaecology, St Michaels Hospital, Southwell Street, BS2 8EG.

Preterm birth is associated with severe perinatal mortality and long term disability.

Rho GTPase are key regulators of the actin cytoskeleton and recent evidence has revealed an up-regulation of Rho A and its effector Rho Kinase (ROK) in pregnant human myometrium. ROK increases myosin light chain phosphorylation and contraction by inhibiting myosin phosphatase, making the myometrium more sensitive to the effects of calcium (calcium sensitisation).We intend to investigate the possible role of Rho GTPases and calcium sensitisation in preterm labour. Immunoblotting and Real-time PCR (RTPCR) to quantify Rho GTPases and related kinases in human myometrium. Rho B, C, D, Rnd2 and Rnd3 proteins are expressed in human myometrium. Expression of Rho B and C mRNA relative to Rho A mRNA is increased in preterm myometrium.

This is the first report of the expression of these GTPases in human myometrium. The difference in mRNA expression between term and preterm myometrium requires further investigation.

Leukocyte density and proinflammatory cytokine expression in human preterm myometrium. I. Osman, A. Young, F. Jordan, I. A. Greer and J. E. Norman. Division of Developmental Medicine, Reproductive and Maternal Medicine, University of Glasgow.

Accumulating evidence supports the view that human parturition represents an inflammatory process. Whether a similar process occurs in the absence of infection during preterm parturition is yet to be determined. The aims of this study were to quantify and compare the total leukocyte population and mRNA expression of interleukin-1β (IL-1β), IL-6, IL-8, cyclo-oxygenase type 1 (COX-1) and COX-2 in myometrium obtained before and during labour. Biopsies of myometrium were obtained from pregnant women delivered by Caesarean section before or after the onset of spontaneous preterm labour. Leukocytes were identified using primary antibodies directed against CD45 and cytokine mRNA expression was quantified using TaqMan technology. Preterm parturition was associated with a significant increase in IL-6 ($p = 0.015$) and COX-2 expression ($p = 0.045$) with an increasing trend for IL-1β and IL-8 mRNA expression. Histological analysis demonstrated no statistical difference in leukocyte density between labouring and non-labouring preterm myometrium. These data lend support to the role of inflammation during preterm parturition.

Prostaglandin E_2 synthesis and prostaglandin E_2 receptors in the pregnant human uterus: a paradox. D. M. Slater,[1] S. Astle,[1] N. Woodcock,[1] M. Vatish,[2] S. Thornton[2] and R. Newton.[1] Biomedical Research Institute[1] Biological Sciences, Warwick Medical School,[2] University of Warwick, Coventry, UK.

Human labour is associated with dramatic increases in prostaglandin synthesis. Prostaglandin E_2 (PGE_2) (used for labour induction) exhibits a particularly wide spectrum of physiological actions depending on which PGE_2 receptors (EP) are present. Stimulation of EP receptors, EP_1 or EP_3 leads to increased uterine contractility, whilst stimulation of EP_2 or EP_4 to relaxation. Regulation of PGE_2 synthesis and differential expression of EP (EP_{1-4}) receptors may be important in controlling uterine activity throughout pregnancy (quiescence) and during labour (contractions). RT-PCR and immuno-histochemistry were utilised to identify and localise PGES and EP receptor isoforms within the pregnant uterus. Myometrial smooth muscle cell cultures and contractility studies were used to assess the functional role of PGE_2. Functional studies identified distinct relaxatory and anti-inflammatory effects of PGE_2. This might explain the variable clinical effects seen with the use of PGE_2. Further elucidation of PGE_2 signalling mechanisms may lead to improved treatments for labour induction.

Preconception prednisolone: a novel treatment for recurrent miscarriage? C. Kalumbi,[1,3] M. Bates,[1] G. Vince,[2] R. Farquharson[3] and S. Quenby.[1] Departments of [1]Obstetrics and Gynaecology and [2]Immunology, University of Liverpool, Liverpool, L69 3BX and [3]Liverpool Women's Hospital, Crown Street, Liverpool, L8 7SS, UK.

No evidence- based treatment is available for idiopathic recurrent miscarriage (RM). Recent studies have shown that high levels of uterine natural killer (uNK) cells are associated with recurrent miscarriage. This study was designed to test the hypothesis that preconception prednisolone reduces uNK cells. Women with at least three consecutive RM were invited to participate in the study. An endometrial biopsy was taken on day 21 of the menstrual cycle. Immunohistochemistry was used to detect (uNK). If uNK cells were high, 20 mgs prednisolone was given orally for 21 days, and biopsy repeated. Of the 60 women recruited, 23 (38%) had high levels of uNK cells. To date, 11 women have had post- treatment biopsy and all have shown a reduction in uNK cell levels. ($P < 0.05$) These preliminary results have shown that preconception prednisolone therapy significantly reduces the levels of uNK cells. Further work is necessary to assess this novel treatment.

Is endothelial-dependent relaxation altered in myometrial vessels taken from women with a history of pre-eclampsia in a previous pregnancy? C. Hall, J. Myers, M. Wareing, J. Gillham and P. Baker. Maternal & Fetal Health